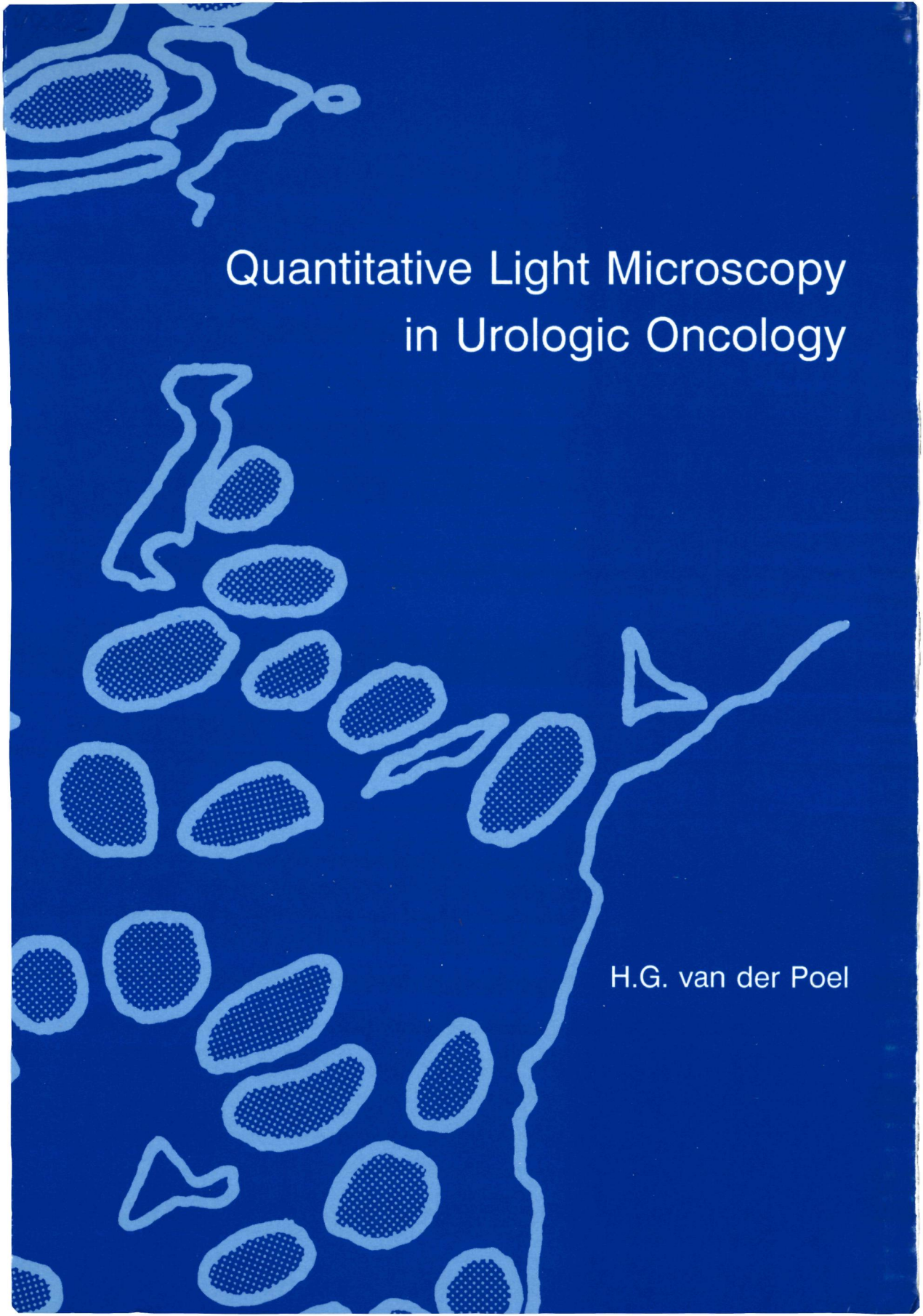
Quantitative Light Microscopy in Urologic Oncology



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Quantitative Light Microscopy in Urologic Oncology

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in het bijzonder de Geneeskunde

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Aan mijn vader en moeder

Contents

List of Abbreviations

2cDI	2c Deviation Index
5cER	5c Exceeding Rate
ANOVA	analysis of variance
BCG	bacillus Galmette-Guérin
CCD	charge-coupled device
ChP	chromatin pattern
CIS	carcinoma in situ
D	difference between highest and lowest feature in tumor
DNA	desoxyribonucleic acid
DNA-MG	DNA malignancy grade
FCM	flow photocytometry or flow cytometry
FNA	fine needle aspiration
FormEll	elongation factor
GMA	glycol methylacrylate
IBAS	image basis analysis system
IOD	integrated optical density
L	lowest value of feature in tumor
M/V	mitotic volume index
MDAH	M.D. Anderson Hospital prostate cancer grading system
MED	median value of feature
MMC	mitomycin
mtDNA	mean total nuclear DNA
mtVOLUME	mean total nuclear volume
N/C	nucleus to cytoplasm ratio
NCr	nuclear crowding
ND	nuclear diameter
NPA	nuclear profile area
NPP	nuclear profile perimeter
NRF	nuclear roundness factor
NuA	nucleolar profile area
OD	optical density
PB	ploidy balance
PCA	prostate adenocarcinoma
PI	proliferation index
PND	proximal nuclear distance
PSA	prostate specific antigen
QLM	quantitative light microscopy
RAM	random access memory
RCC	renal cell carcinoma
SCM	static photocytometry
SD	standard deviation
SPSS	statistical package of social sciences
TCC	transitional cell carcinoma
TURT	transurethral resection of tumor
TURP	transurethral resection of prostate
U	highest value of feature in tumor
UICC	Union International Contre le Cancer
WHO	World Health Organization

Glossary

Term	Meaning
Contexture analysis	Quantitation of low-resolution distribution patterns
e.g.,	
1. proximate nuclear distance	Distance between the nuclear center and that of its nearest neigbor
2. nuclear crowding	An approximation of the ratio of nuclear area to interstitial or cytoplasmic area defined by $4 * NPA / (\pi PND^2)$
Cytometry	Quantitation of cellular features
Feulgen staining	Stoichiometric DNA staining with Schiff's reagent[128]
Flow cytometry (FCM)	Single-cell analysis of cells and nuclei in suspension
High-resolution techniques	Quantitation at higher magnification (e g, 100x objective), low relative pixel size enables detailed analysis of e g, chromatin texture
Histometry	Description of histologic tissue structure
Karyometry	Quantitation of nuclear features·
1 Morphometry	description of size and shape e g
	nuclear roundness factor (NRF)

$$\text{- NRF} = \frac{4\pi \times NPA}{NPP^2} \text{ (Intr ref 176) or } \frac{NPP / 2\pi}{(NPA / \pi)^{1/2}} \text{ (Intr ref 178)}$$

NPA = nuclear profile area
NPP = nuclear profile perimeter

2. Densitometry	Staining intensity or static cytophotometry (e g, DNA content)
3. Texture analysis	Quantitation of high-resolution distribution patterns (chromatin structure) with co-occurrence matrix techniques, run length distribution, etc..
Low-resolution techniques	Quantitation at lower magnification (e g, 20x objective) which results in large relative pixel size as in histometry and cytometry
Mitotic Index (M/V Index)	Expression of mitotic activity as number of mitotic figures per square millimeter of neoplasmic tissue in microscopic field (Intr ref 166)
Static cytophotometry (SCM)	Densitometric measurements for e g, for DNA content quantitation

SCOPE OF THE THESIS

Since the development of light microscopic analysis of oncological processes at the end of the last century, tumor grading proved to be a powerful diagnostic and predictive tool. The visual grading of tumors arose from years of practical experience and has its roots in the empirical analysis of tumor material. In grading, tumors are scored on tumor characteristics for which etiology is ill understood. It is purely descriptive.

The multiparameter nature and empirical approach resulted in a low compliance of grading systems among pathologists. As discussed in the introduction of this thesis, the use of quantitative analysis of light microscopic images can help to grade tumors in a reproducible way. Moreover, quantitative analyses can shed light on biological tumor features such as heterogeneity and tumor development. Hence, quantitative tools serve a wide field of purposes.

The aim of this thesis is to study the potential of quantitative analysis for the grading of urological tumors. Several karyometric features will be tested. A panel of known features is designed including morphometric, densitometric, and texture features, to enable comparison with earlier studies.

The **Introduction** presents a review of the literature on the use of quantitative microscopy for urological tumors.

In **Chapter 1** two studies are presented. Cytomorphometric measurements of bladder wash material is conducted analyzing nuclear profile area. Several material processing methods are applied to study the influence of embedding and staining techniques on nuclear appearance. In the same Chapter a multivariate karyometric approach to grading of bladder cancer is presented.

The reproducibility of interactive and semi-automatic karyometric analyses is commented upon in **Chapter 2**. An answer is sought to the question whether subjectivity, hampering visual tumor grading, also plays a role in karyometric studies and can be annulled in the latter technique.

Whereas the first two Chapters deal with correlation with visual tumor grading, in **Chapter 3** and **Chapter 4** two studies are presented on the clinical application of karyometric analysis. In **Chapter 3** nuclear features are studied in histological material of recurrent superficial bladder cancer for the prediction of tumor progression. A clinical approach to the quantitative follow up of superficial bladder cancer patients by karyometric analysis is described in **Chapter 4**. In order to use the developed

system in routine settings, we studied the logistics of material sampling and reportage in this study as well.

In the following two Chapters, **Chapter 5** and **6**, karyometric analysis is applied for the grading of renal cell carcinoma. Regarding extensive intra-tumor heterogeneity, different tumor areas are karyometricly analyzed to come to a quantitative description of variation in intra-tumor nuclear phenotype.

The final study deals with karyometric analysis of prostate adenocarcinoma (**Chapter 7**). Multiple tumor loci within the prostate are analyzed and karyometric data are compared to tumor location and pathological tumor stage. The heterogeneity of karyometric features in different tumor locations is studied in different stages of local progression in organ-confined disease.

Overall we aim at applying quantitative light microscopic techniques for the analysis of tumor biological characteristics in urological cancers. Special attention will be paid to the clinical applicability of the methods studied.

QUANTITATIVE LIGHT MICROSCOPY IN UROLOGIC ONCOLOGY

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1 INTRODUCTION

Prognosis in cancer depends on three factors, as stated by Mostofi[1]: *'the response of the patient, the commissions and omissions of the physician, and the potentialities of the neoplasm'*. In 1858, Virchow discussed the microscopic changes that take place in tumor cells and their nuclei. The morphological characteristics of tissue, cell, and nucleus proved to reflect malignant behavior and were used to evaluate neoplasms with several grading systems. Here, we will give an overview of the different grading systems used in uropathology in historic perspective.

Low reproducibility and the inability to quantitate features by visual analysis render the grading systems less applicable. Digital images analysis of tissue and cell characteristics in microscopic images is used to study quantitatively the histologic and cytologic changes of neoplasms in an objective and reproducible way. In line with this we discuss the literature on quantitation of microscopic features of prostatic adenocarcinoma, transitional-cell carcinoma of the bladder, and renal-cell carcinoma and we dwell on the pitfalls of the use of quantitative light microscopy (QLM) in routine uropathology.

2 VISUAL GRADING OF UROLOGICAL TUMORS

2.1 Grading of Prostate Adenocarcinoma

The number of grading systems proposed for prostate adenocarcinoma outnumber those for renal and bladder tumors. The first grading system for prostate cancer was introduced by Broders in 1925[2] and was based on the estimated percentage glandular differentiation. Many grading systems followed but did not always show significant correlation with survival.[3,4,5,6] On the other hand several grading systems showed low reproducibility or were not tested for it. Three histologic features correlated with prognosis; 1. tumor volume, 2. glandular differentiation, 3. nuclear anaplasia. The four most commonly applied grading systems for prostate adenocarcinoma will be discussed here. In table 1 an overview is given of the literature on grading of PCA. For each study is indicated whether glandular differentiation, nuclear anaplasia, or both are included.

In 1975 Mostofi[7] introduced a grading system based on the nuclear anaplasia and glandular differentiation of the tumor glands. Several studies used this system and found it useful in assessing tumor prognosis.[8,9] However, other studies found the Mostofi grading system less useful to identify high risk patients.[10,11]

Besides the Mostofi system the Gleason system[12] is widely used. The system graded prostate adenocarcinoma according to four tumor growth pattern characteristics: glandular size and shape, glandular differentiation, and invasion in stroma. The two most predominant patterns were recorded and their values added to arrive at a grade ranging from 2-9. Gleason found the reproducibility of the grading system about 80%, others found lower rates varying from 50 to 70%.[9,10,13,14] Conflicting results were obtained when Gleason score was used to predict lymph node metastases.[15,16,17] Fine needle biopsies graded according to the Gleason system showed significant variation when compared to the prostatectomy specimens.[18,19,20]

In 1982 Böcking and associates[21] proposed a grading based on glandular differentiation and nuclear anaplasia. The part of the tumor with the highest grade determined tumor grade. The intraobserver reproducibility of one of the authors was assessed in this study and was found 87.5% for the entire system. The reproducibility for the cytology part (nuclear anaplasia) being lower than for the histology part (glandular differentiation). In other hands the Böcking grading system showed 75% agreement between three pathologists.[9]

Brawn and associates (1982)[11,22] objected to the grading systems of Mostofi and

Gleason and attempted to improve both the reproducibility and simplicity of grading by developing a low-power microscopic examination method, the M.D. Anderson Hospital (MDAH) system. Initially four grades were used, likewise Broders classification, based on the percentage of tumor that forms prostatic glands. Since grade 2 and 3 did not seem to have prognostic significance these two grades were combined.

Schröder and associates (1985a,b,c)[23-25] in collaboration with Mostofi attempted to replace "grade" by a single grading feature in order to simplify the Mostofi grading system. A retrospective study using multivariate analysis including several grading features showed that three independent features were of significance: architectural features, mitotic activity and nuclear anaplasia. The grading resulted in the subdivision of 5 classes of the patients with different outcome.

De las Morenas and associates (1988)[9] compared four grading systems (Gleason, Mostofi, Böcking, MDAH) and concluded that the Böcking system was preferable considering both reproducibility and correlation with stage. The MDAH-system proved to have the best reproducibility, but lacked a good correlation with stage. Unfortunately in this study no correlation between grade and survival was investigated. Grading of tumor cells in transrectal biopsies showed even lower values for reproducibility in different studies varying from 50% to 60%.[26,27,28]

Table 1. Visual grading of histologic and cytologic prostate adenocarcinoma and prognosis in different studies*

study	Grading system glandular diff.	nuclear anaplasia	predictive value	material	ref.nr.
Muir (1934)	+	+	high	h	[5]
Kahler (1938)	+	+	high	h	[3]
Pool and Thompson (1956)	+	+	high	h	[6]
McNeal (1965)	+	+	none	h	[4]
Gleason (1966)	+	-	high	h	[12]
Utz and Farrow (1969)	+	+	none	h	[260]
Esposti (1971)	-	+	high	c	[188]
Esch *et al* (1972)	+	+	none	h	[261]
Mostofi (1975)	+	+	high	h	[7]
Epstein and Fatti (1976)	+	+	high	h	[262]
Barzell *et al* (1977)	Gleason		none	h	[15]
Harada *et al* (1977)	Gleason		low	h	[10]
	Mostofi		low	h	
Kern (1978)	Mostofi		high	h	[8]
Voeth *et al* (1978)	-	+	low	c	[28]
Murphy and Whitmore (1979)	Gleason		low	h	[14]
Kramer *et al* (1980)	Gleason		none	h	[17]
Gaeta (1981)	Gleason		low	h	[16]
Böcking *et al* (1982)	+	+	high	h	[21]
Brawn *et al* (1982) (MD Anderson)	+	-	high	h	[11]
Catalona *et al* (1982)	Gleason		low	c	[18]
Böcking (1983)	-	+	low	c	[26]
Lange and Narayan (1983)	Gleason		low	c/h	[263]
Garnett *et al* (1984)	Gleason		high	c	[20]
Schröder *et al* (1985c)(Mostofi/ Schröder)	+	+	high	h	[25]
Mills and Fowler (1986)	Gleason		low	c/h	[264]
Layfield *et al* (1987)	Esposti / Gleason		high	c/h	[189]
de las Morenas *et al* (1988)	Gleason		low	h	[9]
	Mostofi		low	h	
	Bocking		high	h	
	MD Anderson		low	h	
Gallee *et al* (1990)	Broders		high	h	[30]
	MD Anderson		high	h	
	Mostofi		low	h	
	Gleason		low	h	
	Mostofi-Schöder		high	h	
Mukamel *et al* (1990)	MD Anderson		high	h	[265]

h = histology; c = cytology

*) A selection of different studies over the years is made This list is not covering all studies on the topic but to the authors opinion, the most important articles are cited.

In 1983, Grayhack and Assimos[29] reviewed the literature on grading and staging of prostate adenocarcinoma and concluded that grading and staging should be familiar to both pathologist and urologist and that one should bear in mind that despite good correlation with prognosis, in some cases these observations may provide no prognostic information.

Gallee and associates (1990)[30] compared the prognostic accuracy of five grading systems (Broders, MDAH, Mostofi, Gleason, Mostofi-Schröder) and did find the Gleason system of minimum prognostic value. The Mostofi-Schröder system showed reasonable predictive power, however the Broders system was preferred considering the low interobserver variation compared to the Mostofi-Schröder system.[31]

Concluding can be stated that grading of prostate adenocarcinoma showed to be in good correlation with survival to some extend for the individual grading systems. Both glandular differentiation and nuclear anaplasia do correlate with malignancy. Low reproducibility though, may render some grading systems less useful. For the grading of tumors by biopsies assessment of grade of the worst part of the material seemed to be most useful,[28] albeit variation in grade compared with prostatectomy material is often high.

2.2 Grading of Transitional Cell Tumors (TCC)

Since Broders (1925)[2] introduced a grading system for transitional cell carcinoma several changes in the grading system have been made. Early grading systems for TCC included the level of tumor infiltration. A correlation up to 80% of tumor grade with pathologic stage have been reported, the predictive value of grade within the same tumor stage however seems to be of less value.[32]

Franksson (1950)[33] described a grading system that combined depth of infiltration with cellular differentiation to classify the tumor in one of seven grades. Using this system 30-40% of the TCC of the bladder were graded "benign" only recognizing malignancy when invasion of the lamina propria had occurred. In contrast with this, and likewise Broders,[2] were the grading systems proposed by Friedman and Ash (1959)[34] and Marshall (1956).[35] These systems regarded a malignant behavior as soon as the urothelium deviated from the normal appearance.

Dean and associates (1954)[36] proposed a grading system using histological arrangement (papillary or nodular), extent of infiltration and tumor size in concert. They compared the original grading (Armed Forces Institute of Pathology) with their review grading. Again the so-called papilloma group showed discrepancies between

the two grading systems. Especially differentiating between high-grade papillomas and low-grade carcinomas. They concluded that both grading systems offered a good prognostic index of tumor behavior especially when the infiltration status was taken into account.

Marshall (1956)[35] found a higher 5-year survival (47%) rate in patients with high-grade and low-stage tumors, than in patients with low-grade and high-stage cancer (15%), both treated surgically. They also discussed the diagnosis of papilloma and stated that, however the overall cure rates of the carcinoma population can be blurred by including papillomas in the grade-1 carcinoma group, this seemed to be warranted considering the more often occurrence of carcinoma in patients with papillomas.

Mostofi (1956)[37] divided 2678 patients in having non-infiltrating or infiltrating tumors of the bladder. The group of patients (332 cases) with non-infiltrating grade 1 TCC showed a 5-year survival of 85%. The survival rate decreased to 20% for patients with infiltrating grade-3 tumors.

In 1965 Bergkvist and associates[38] described a grading system based on pattern of growth as well as cellular and nuclear morphology. Referring to "cellular deviation" when deviation of the cellular pattern from normal urothelium is to be described. They found a clear distinction between survival in the grade-0 and 1 group and in the grade-2 to 4 group.

The most widely used system was presented by Mostofi in 1973 (WHO).[39] The protocol contains three grades characterized by difference in anaplasia. Whether the simplicity of this system adds to its objectivity was doubted.[40,41]

In 1979 Collan and associates[42] stated that clinical staging would be more effective than histological grading, and they suggested that grading should be used to complement clinical staging. Ooms and associates (1983)[43] investigated the reproducibility of the histological grading (WHO) of TCC. Disturbingly low inter and intra-individual consistency was found in a group of five pathologists.

The grading according to the WHO standards resulted in an equal distribution of tumors over the three tumor grades.[36,39,42] An increase with tumor stage however, was accompanied with a increase in tumor grade.

Besides histological grading, urinary cytology enables assessment of the presence of TCC. Papanicolaou and Marshall (1945)[44] described a technique of sampling cells from urinary specimen. Grading systems for urinary cytology are based on the cellular and nuclear features.[36,45,46,47,48,49,50,51] However, screening for cancer appeared to be less successful and cytology seemed to be more useful in detecting recurrent tumors.

Whereas low-grade lesions have a detection rate of approximately 60%, high-grade lesions could be readily detected even with negative cystoscopic findings.[52,53,55] Studies on the reproducibility of cytological tumor grading, however, showed results similar to the low intraobserver and inter consistency found in histological grading.[58]

A specific type of TCC should be mentioned here. Melicow and Holowell (1952)[56] described the intraurothelial lesions in a macroscopically normal bladder. The cells of these so-called carcinomas in situ (CIS) were often of high-grade malignancy[46] and approximately fifty percent of the patients developed invasive carcinoma within five years of diagnosis.[57,58] Boon and associates (1986)[59] indicated the importance of cytology in these patients: histology may be false-negative due to denudation as a result of excessive exfoliation of the malignant CIS cells.

In summary, the grading systems for TCC, though useful in several studies, clearly lack consistency. The staging of TCC was found to be of more predictive value than grade. Though differentiation of TCC as assessed in light microscopic analysis has prognostic value, a reproducible and consistent grading system is still lacking.

2.3 Grading of Renal Cell Carcinoma (RCC)

Since Grawitz[60] described the RCC in 1884 and found its resemblance to the adrenal cortex, many studies discussed origin and prognosis of this tumor. The renal origin of the tumor was supposed by Stoerk in 1908.[61] In 1944, Melicow[62] found that patients with granular cell renal tumors had a worse prognosis compared with the clear cell tumors. Other studies confirmed these findings.[63-72] Several studies, however, were not able to find a correlation between cell type and malignant behavior.[73-80] An explanation can be the frequent presence of multiple cell types within one tumor.

An other division of RCC is in papillary and non-papillary growing tumors. In a multiparameter analysis Delahunt and associates (1987)[80] did not find correlation between the histological pattern and prognosis. A clear correlation was not to be expected since only 77% of tumors showed heterogeneity in histological pattern. Geneticly, however, the two histological types were found to be distinct.[81]

Like prostate and bladder cancer tumor grade seemed to offer merely additional prognostic value to tumor stage and the presence of metastases.[62-67,70-72,74,78,82-90]

The grading of RCC can be divided into two kinds of systems. One, the general impression of the grade of anaplasia in the tumor material. The second kind of grading systems is based on cell nuclear characteristics.

The present review will discuss the two kinds of systems separately. Nuclear

grading will be discussed in particular since image analysis so far is mainly concerned with nuclear features. Considering the large variability in the investigated patient populations and the many different grading systems used it is not warranted to compare the results of the different studies. Hence, the main problems and findings will be discussed in short for some of the studies. In general it might seem clear to the reader that with an increase of grade the prognosis worsens.

The first grading for RCC on base of the cellular differentiation was proposed by Hand and Broders in 1932.[67] They drew up four grades of malignancy and found a decrease in post-operative life expectancy with an increase of tumor malignancy. Their grading was based on histological as well as cytological features. In an even larger population Priestley (1939)[88] used this grading system and concluded that the system could be useful in assessing the general percentage of survivals, however, it contained insufficient evidence in itself on which to base an accurate prognosis in a single case. In the years following many authors proposed their own grading systems.[67,91-93] Mathisen and associates (1965)[87] found grade to be one of the most important prognostic factors. In 1965 Arner and associates[73] classified RCC in low and high degree of malignancy and an intermediate group. They found the degree of cellular differentiation the best predictor of prognosis. The grading system by Arner was used in several studies.[71,74,88,94,95] In 1978 Syrjänen and Hjelt[95] found different survival rates for the different grades compared to the data in Arner's study[73]. The overall 5-year survival rates of both studies, however, did not differ. Syrjänen and Hjelt suggested that this was due to a lack of reproducibility of the grading as described by Arner and associates (1965).[73] Selli and associates (1983)[71] found lower tumor grades in patients without metastasis at time of diagnosis. The prognosis of the group of patients without metastasis in this study was again closely related to tumor grade.

In 1971, Skinner and associates[72] introduced a grading system that was based on nuclear features alone. In this study the grading was mainly based on the visual interpretation of the size of the nuclei present. Tumor grade increased with an increase in nuclear size. Also in contrast with the grading system of Hand and Broders (1932)[67] is that not the general impression determined grade, but that grade was defined by the area with highest grade, present in the tumor. In the same study they found a good correlation of nuclear tumor grade (based on anisonucleosis, chromatin pattern, nucleoli and mitotic figures) with survival. However, the majority of patients had tumors in the intermediate category, thus limiting the classification value of this system.

Fuhrman and associates (1982)[83] studied the significance of stage, grade, tumor size, and cell type and found nuclear grade the best predictor of distant metastasis. Grade-1 tumors only metastasized in 9% of the patients. They found best disease free survival in patients with oncocytomas compared to other cell types. Grade-2, 3, and 4 did not show significant difference in metastasis rate. Correlation of grade with survival was significant. Grade-1 tumors showed highest survival rates, grade-2 and 3 intermediate, and grade-4 cancer correlated with low survival. Although survival was similar for grade 2 and 3 tumors, these two grades were morphologically quite distinct. The authors did not discuss a reason but suggested study on larger populations. On the other hand there appeared to be large difference between stage 1 grade 1 and grade 2,3,4 tumors as far as malignancy was concerned. The frequency of metastases within the latter group was not different. This again reduces the stratification capabilities of the system by only differentiating high and low grade malignancies.

The grading system according to Fuhrman and associates[83] was also used in a study by Medeiros and associates (1988).[79] No significant difference was found in survival between grade 1 and 2 as opposed by the study of Fuhrman and associates (1982).[83] They concluded that nuclear grade rather than cell type may be important for determining prognosis.

Summarizing the results of the different grading systems, the data suggest that nuclear grading rather then general histological classification is important for assessing prognosis in RCC. This is a promising result considering the simple grading criteria in the nuclear grading systems. Despite a higher reproducibility of the nuclear grading systems limited stratifying power is to be expected.

3 QUANTITATIVE PATHOLOGY

The changes associated with atypia or tumorous growth can often be perceived with microscopic grading techniques. Description of the changes led to the development of grading systems in which histologic, cellular, and nuclear features were described and related to malignant potential. However, as described in the preceeding part of this chapter the descriptive, often subjective nature of the grading systems resulted in low reproducibility.[30,31,43,96,97] As opposed to the subjectivity of visual assessment of tumor properties in light microscopy, quantitation of nuclear, cellular, and tissue architectural characteristics, can provide a more accurate and reproducible description of microscopic images of tumor tissue.

Several techniques can be used for quantitation. Visual estimation of size based on references within the image and point-counting techniques are simple and inexpensive methods for quantitation of a small number of features. Flow cytometry (FCM) is a commonly used technique to assess DNA content of cell nuclei. Digital image analysis permits relatively inexpensive and fast multiparametric analysis of microscopic images. It can also provide extra information about the tumor cell not perceptible by the human eye, such as DNA content, high-order texture description, and specific shape irregularities.

Quantitative light microscopy (QLM) includes histometry (description of tissue architecture) and cytometry and karyometry (measurement of cellular and nuclear characteristics respectively). Results of QLM in measuring DNA content cannot be interpreted without referring to the vast number of FCM studies on urologic tumors, so the results of FCM studies will be briefly reviewed as well.

3.1 Quantitative Light Microscopy Features

After digitization i.e., pixel-wise recording with a video camera and translation of grey values into 8-16 bits computer memory, the objects (cells and nuclei) can be detected (segmentation). The features used to describe the cellular (cytometry) and nuclear characteristics (karyometry) after segmentation consist of three groups (Figure 1): 1. morphometric, related to size and shape of objects; 2. static cytophotometric (SCM), related to staining intensity (in the case of Feulgen staining, refers to quantitation of nuclear DNA content); 3. and textural and contextural, related to chromatin pattern; and to cellular and nuclear arrangements, respectively.

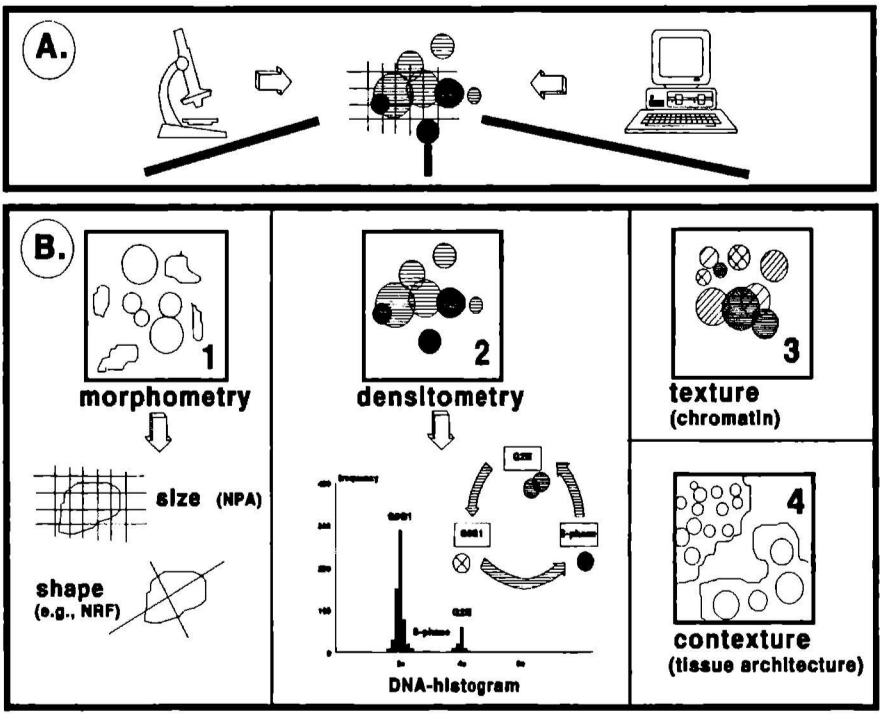

Figure 4. Features studied in quantitative light microscopy.

3.1.1 Morphometry

Morphometric features describe size and shape of objects. Size can be measured relatively simply by counting the number of pixels occupied by the object and calibrating for magnification. Description of shape demands a more complicated mathematical approach. Before starting an entire panel of shape descriptors, one has to determine which shape needs to be described. Two commonly used shape descriptors are the elongation factor (FormEll) and the nuclear roundness factor (NRF). FormEll is the ratio of the minor to the major axis of the object. NRF is a ratio of area to perimeter (see Glossary). FormEll is 1 for a circle and smaller than 1 for an ellipse. NRF decreases when an object deviates from a circle, as is the case in an ellipse as well as in an irregularly-shaped round object. Hence, NRF confuses roundness and circularity[98] and so is less useful as a shape descriptor. Several authors

have described this problem and suggested other descriptors of shape, with the inevitable pitfalls of using them.[98,99]

3.1.2 Static Cytophotometry vs Flow cytometry

A common method for quantitating DNA content in cell nuclei is flow cytometry,[100,101,102,103] which uses nuclei in suspensions. In QLM, DNA content is measured in a similar way, except that the nuclei are on slides[104,105] instead of in suspension - the technique is called static cyto(photo)metry (SCM).[101,106] SCM can be applied on cytologic as well as histologic material; smears,[107,108,109,110,111] cytospin preparations,[112,113,114] or tumor imprints[101,115,116,117] obtained from either paraffin-embedded archival material,[118,119,120] fresh-fixed tissue,[101] or urine[112] allow more accurate measurements of DNA content than sections of embedded histologic material.[101,107] DNA-content measurements in histologic sections, however, were applied in several studies[121,122,123,124,125] and proved to be useful,[110] and the substantial loss of cell populations by disaggregation of paraffin-embedded archival material[113,118,119,120] implies substantial benefit of SCM on histologic slides.

For SCM, stoichiometric DNA staining according to Feulgen is generally used.[101,107,126] With Feulgen staining, the integrated optical density per nucleus correlates with DNA content. Fluorescent staining techniques, as with acridine orange and DAPI, can also be used to quantitate DNA content.[126]

SCM and FCM seem to have different specificities; SCM recognizes structural alterations, whereas FCM describes minor DNA-content alterations in the tumor stem-line more exactly.[106,108] Another important difference between FCM and SCM is the inability of nuclear visual selection in FCM. SCM offers the pathologist the opportunity to preview or review the measured objects: for each nucleus or cell, both data and microscopic images can be stored. That not only reduces noise in measuring specific objects, but can also assist the pathologist during screening of the slide. However, the number of cells needed for analysis is much higher in FCM than SCM and that favors SCM, especially in the analysis of histologic slides or when sampled material contains only few nuclei (e.g., prostatic fine needle aspirates, urinary cytology, and renal biopsies) (Table 2).

Table 2. Advantages and disadvantages of flow cytometry (FCM) and static cytophotometry (SCM).

Characteristic	FCM	SCM
Application	DNA content and other markers	DNA content and other markers
Material	Cell suspension	Cytology and histology
Tissue processing	Elaborative (single cell suspensions)	Simple, when routine material used
Amount of material needed	Large (approximately 10,000 cells), no visual selection of the objects possible	Small FNA or biopsy material can be analyzed, visual interpretation still possible
Accuracy	High, but debris and clustered nuclei might produce noise in histogram	Coefficient of variation often higher, DNA index less accurate
Sensitivity	High for minor shifts in DNA content, but, cell populations with few cells might be missed	High, especially with few of aneuploid cells (selection)
Reproducibility	High	High, but dependent on selection criteria and sampling size
Sample preservation	Hours because fluorescent staining techniques are used	Months to years, when Feulgen staining is used (provided stored properly)
Costs	High	Moderate
Measuring time (per sample)	Minutes	Minutes to hours, dependent on computer used

3.1.3 The DNA histogram

SCM and FCM both result in a DNA histogram, a representation of the distribution of cells in the tissue in the different phases of the cell cycle. The cell cycle can be divided into three basic compartments: the G_0G_1 phase or resting and non dividing phase; the S phase, or synthesis phase; and the G_2M phase in which nuclear DNA is doubled and cell division takes place (mitosis). The peaks in a DNA histogram,

whether obtained with FCM or with SCM, reflect the distribution of cells in the compartments of the cell cycle. A normal dividing cell has a diploid (2c) peak (which represents the G_0G_1 phase), a tetraploid (4c) peak (with the cells in the G_2M phase having twice as much DNA as normal DNA), and some cells between these peaks (S phase).

On the basis of a DNA histogram, tumors are divided into diploid and aneuploid. Diploidy indicates that the mean peak in the DNA histogram is around the 2c region and the DNA pattern resembles the distribution of cells over the cell-cycle phases of a normal cell population. Aneuploidy, or nondiploidy, refers to DNA histograms with main peaks outside the 2c region or the presence of one or more abnormal peaks in DNA distribution. The definition of diploid and aneuploid, however, is not always clear. Besides the place of the main peak related to the 2c region, the percentages of cells in the different peaks also determine ploidy. A normal cell population is characterized by a diploid pattern with approximately 90% of cells in the 2c peak.

Notwithstanding the definitions of ploidy patterns, a subset of DNA histograms will still be difficult to interpret visually.[103] Table 3 shows some algorithmic approaches to DNA histograms obtained with SCM. Tumor heterogeneity, i.e., several cell populations with different DNA content within the same tumor, resulting in very close or overlapping peaks in the DNA histogram may render histogram interpretation difficult. The finding of 'a large diploid' tumor may be caused by inadequate sampling of material: the aneuploid tumor cell populations are missed.

Table 3. Interpretation of DNA histogram obtained by SCM or FCM.

	abbrev.	name	remarks
algorhitmic interpretation			
Böcking et al. [256]	2cDI	2c Deviation Index	deviation of 2c reference value
	5cER	5c Exceeding Rate	cells > 5c minus 2c exponents
	DNA-MG	DNA-malignancy grade	1.757 * log(2cDI+1)
Opfermann et al. [257]	PB	Ploidy Balance	difference euploid and aneuploid
	PI	Proliferation Index	% of cells between peaks
Koss et al. [103]	DI	DNA Index	place of peak mean relative to 2c
	CV	Coefficient of Variation	peak tightness (SD/mean).
visual interpretation			
Auer et al. [258]	diploid	I	normal histogram, <0.5 % more than 5c
		II	G_2M > 15%, more 2% of cells over 5c
	aneuploid	III	abnormal $G_{0G}1$ exceeding control with > 2xSD
		IV	wide range of DNA values
Forsslund et al. [201]	D-type	diploid	all cells lower than 2.5c or inbetween 3.5c and 4.5c
	T-type	tetraploid	like D-type but with a higher tetraploid peak
	A-type	aneuploid	non D or T type

To determine the normal 2c value in the DNA histogram, a reference is needed. As a reference for diploidy several cells are used: trout red blood cells,[114] fibroblasts, endothelial cells,[101] lymphocytes,[110,127] and small phosphor particles in fluorescence techniques.[128] Reference material should be stained at the same time as the test material, and differences in hydrolysis time related to differences in chromatin structure should be taken into account[128] when Feulgen staining is used.

SCM is useful for assessing DNA content, especially when only little cytologic material is available. The most accurate DNA values can be measured in smears or imprints. However, loss of cell populations can be a major problem when archival material is used. For correction of histograms obtained in histological material several mathematical approaches have been suggested.[129]

3.1.4 Texture and Contexture Analysis

Various approaches to chromatin-pattern analysis have been extensively discussed elsewhere[130,131,132] and have been shown to be useful in the discrimination of the phases of the cell cycle[133] and in quantitation of therapy effect.[134] Features used are based on the co-occurrence matrix[130,131,132] or on run-length distributions.[132]

Tissue architecture (context) in histologic slides (histometry) can be described with features like cell density or glandular size in prostatic cancer. Similar features are used for cell-group analysis in cytology.[135] Furthermore, histometry is used to describe cyto architectonics e.g., the epithelium to stroma ratio,[136] glandular cellularity in prostatic tissue,[137] location and polarity of nuclei in stratified epithelium,[138] and macroscopic analysis of the number of tumor-containing prostatic chips in material from transurethral resection.[139]

3.2 Material Preparation Techniques

Fixation[107,140,141,142] and other processing techniques[106,122,143] play an important role in both cytology and histology. Hence, standardization is obligatory. Routine formalin fixation for histology and ethanol-based fixation for cytology can be used. Whereas the use of sectioned material makes it necessary to measure slices of objects, smear or cytospin techniques require more complicated processing techniques and entail the loss of histologic context and the selection of some cell populations with enzymatic or mechanical disaggregation methods.[113] Feulgen-Schiff staining, a relatively easy-to-use 3-hour staining procedure is generally applied for karyometric analysis.

For histometry,[144,145,146] not only routine paraffin embedding, but also plastic embedding, resulting in more constant and thinner sections, can be used.[122,143] The section thickness to use depends on the size of the objects to be measured.[140,143,144,147] To recalculate the data obtained from sectioned material to the three-dimensional domain, several stereologic approaches are available.[98,129,148,149,150] No study has yet compared the stereologic approaches, but results indicated that DNA histograms obtained in histologic sections lack the information provided by SCM-derived DNA histograms of cytologic material.[106]

3.3 Quantitative Microscopy and Immunohistochemistry

Besides DNA analysis, cytophotometry can be applied to the quantitation of immunohistochemical stains. Antigens specifically detected by antibodies and visualization with labeled secondary reagents can be quantitated. Acquisition of images at different wavelengths allows measurement of antigen expression per nucleus[151] or as a percentage of tissue area.[152,153,154] Specific staining techniques can be used for quantitation of markers of proliferation, such as: mitosis frequency,[155,156,157] bromodeoxyuridine,[158,159] and Ki67 labeling techniques.[151] Chromosomal changes detected with recently developed interphase cytogenetic techniques can also be quantitated with image analysis techniques.[160]

3.4 Quantitative Microscopy Systems

Developments in computer technology have enabled fast and precise measurements of objects in microscopic images. Several image-analysis systems have been developed according to the following principles. The image is recorded with a CCD videocamera and stored in computer memory (RAM) or on other storage media. After shading correction,[155] the image is segmented to define objects of interest.[161,162] That can be done with manual contour drawing (interactive system) or with segmentation algorithms. In an interactive, or open, system, the operator selects the objects to measure. Automated systems use a decision scheme to identify objects of interest.[163,164]

Reproducible measurements can be obtained with interactive systems.[97,165-171] Selection of objects to measure, however, can be a major reason for inconsistency.[165,168,169,171,172] Ooms and associates[169] introduced a histomorphometric selection technique for transitional cell cancer (TCC) based on specific parts of the tumor as assessed in the visual grading. They distinguished three cell groups in the tumor: superficial cells, deep cells, and cells with large nuclei. Although simple and reproducible, this technique is less applicable to nonpapillary growing tumors. Selective cytomorphometry, measuring nuclear profile area has correlated well with cytologic grading and has proved to provide more information than histomorphometry in TCC samples alone.[170] Considering the large variety of nuclei present in the tumor, it is mandatory to define clearly the parts of the tumor used for analysis. Histomorphometry performed in this way has shown a correlation between visual grading and QLM and has proved to be more predictive than measurements of

randomly selected nuclei.[97,166-171]

Collan and associates[172] discussed the difference between diagnostic and investigative interactive-morphometry. They concluded that, as analyses in investigative morphometry are more standardized, the number of objects to be measured when morphometry is applied as a diagnostic tool is often much larger for the same level of accuracy.

4 QUANTITATIVE MICROSCOPY AND UROLOGIC TUMORS

4.1 Prostatic Adenocarcinoma (Table 4)

4.1.1 Morphometry

4.1.1.1 Nuclear size

Since the first report of the correlation between nuclear size and tumor grade in prostatic adenocarcinoma (PCA),[173] several studies have found correlation between the degree of differentiation and distributions of nuclear size in fine-needle aspirates (FNA)[173] or biopsy material.[137,175,176] Unfortunately, no characteristic pattern of diagnostic criteria was found either for prostatic cancer cells in general or for the different tumor grade in cytologic material from the prostate.[175] Blom and associates[176] empirically found a cutoff value of 34% for the coefficient of variation of nuclear size (V_{area}) measured in histologic slides to be of prognostic value in prostatectomy specimens. However, sensitivity and specificity of the test were only moderate. In general, the nuclear size and especially its coefficient of variation seem to increase with tumor grade. High values correlate with a decreased survival rate, but, many false-positive and false-negative results render this feature alone less useful for routine clinical use.[177]

Table 4. Morphometry, static cytophotometry, and (con)texture analysis in prostate adenocarcinoma.

	material (nr)	parameter NRF	NPA	NuA	Npo	Ell
Morphometry						
Stöber and Schmidt[173] (1980)	1.(71)		g+			
Diamond and associates[178] (1982)	1.(17) stage B2	p+	p0			
Spaander and associates[174] (1982)	5.(6)		g+			
Tannenbaum and associates[191] (1982)	1.(52)			p+		
Böcking and associates[175] (1984)	5.(52)		g+		g+	
Epstein and associates[180] (1984)	1.(19) stage A2	p+				
Paulsen and associates[185] (1986)	1.(105)	p0				
Amberson and associates[184] (1987)	5.(34)	p0	p+		p+	
Clark and associates[187] (1987)	5.(26) st. A-B	p0				
Eichenberger and associates[179] (1987)	1.(20)					p+
Mohler and associates[181] (1988)	1.(15) st. A-B	p+				
Partin and associates[182] (1989)	1.(18)	p+				p+
Hutchinson and associates[183] (1989)	5.(26) stage A2	g0	g+		g+	
Tritz and associates[188] (1989)	5.(?)	g0	g+			
Bibbo and associates[204] (1990a)	1.(30)		g+		g+	
Blom and associates[176] (1990)	1.(2					
Petein and associates[177] (1990)	3.(39)		g+			
Schultz and associates[135] (1990)	5.(35)	g0	g+			
Densitometry						
grade		ploidy		bad	prognosis/high	
Sprenger and associates[117] (1974)	5.(32)	g-				
Zetterberg and associates[199] (1976)	5.(33)	g+		hetero- aneuploidy increased with grade		
Böcking and associates[175] (1984)	5.(52)	g0				
Seppelt and associates[200] (1984)	5.(80)	g+		2cDI, 5cER, ploidy rate		
Seppelt and associates[203] (1986)	5.(85)	g+		automated grading not possible		
Amberson and associates[184] (1987)	5.(34)	p0				
Böcking and associates[109] (1988)	5.(52)	p+		DNA-malignancy grade		
Bibbo and associates[137] (1990b)	1.(30)	g+		aneuploidy (5cER)		
Forsslund and Zetterberg[201] (1990)	5.(213)	p+		A-type (aneuploid) histogram		
Petein and associates[177] (1990)	3.(39)	g+		aneuploidy		
Peters and associates[202] (1990)	1.(44) st. A-B	p+		aneuploidy		
Stenkvist and associates[111] (1990)	5.(22)	g0				
Greene and associates[125] (1991)	1.(30)	g+		peripheral zone non-diploidy at lower volume		

Table 4 (continued)

(Con)texture analysis			
		ChP	Contexture
Böcking and associates[175] (1984)	5.(52)	g0	g+
Hutchinson and associates[83] (1989)	5.(26)	g+	g+
Bibbo and associates[204] (1990a)	1.(15)	g+	g+
Bibbo and associates[137] (1990b)	1.(30)		g+
Schultz and associates[135] (1990)	5.(35)	g+	g+

(material 1. paraffin section
2. smear/cytospin
3. imprint
4. plastic (GMA) section
5. fine needle aspirates/biopsies
6. urine

correlation:
p= prognosis
g= tumor grade
s= tumor stage
d= DNA ploidy
+= pos; -=neg; 0=no correlation

features:

NRF	=	nuclear roundness fator			
ND	=	nuclear diameter			
NCr	=	nuclear crowding			
PND	=	proximate nuclear distance			
FeII	=	ellipticity by Fourier analysis			
N/C	=	nucleus/cytoplasm ratio	EII	=	formELL (minor/major axis)
NPA	=	nuclear profile area	SDA	=	SD of nuclear profile area
NuA	=	nucleolar profile area	ChP	=	chromatin pattern
Npo	=	nuclear polymorphism	M/V	=	mitotic index (Table 1)

4.1.1.2. Nuclear shape

In several studies, a correlation of interactively measured nuclear roundness factor (NRF)[178-182] and other descriptors of nuclear shape[179,181-183] was found with clinical prognosis. Other studies, however, could not confirm the findings.[137,176,184-187] The reasons for the discrepancy, with NRF as a prognosticator for PCA, can be:

- differences in material processing,
- differences in analytic techniques, and
- differences in patient and material selection.

Although Epstein and associates[180] found a correlation between the NRF measured in FNA and histologic material, studies using FNA material did find NRF a useful predictive tool in PCA,[109,187] in contradiction to studies on histological material.[178-182] The use of paraffin-embedded material also did not result in uniform recognition of the predictive value of NRF.[176,185] Section thickness, fixation, and staining techniques were not similar in different studies, but difference among them are unlikely to have caused the discrepancies.

That the inconsistency of the value of the NRF was due to differences in measuring equipment is made less likely by the findings of Diamond and associates:[178] NRF was a useful prognosticator in A2, B1, and B2 tumors, but in a larger population of patients with prostatic cancer, neither Diamond nor Blom and associates[178] found it a useful prognosticator. Clark and associates[187] suggested that NRF is of prognostic value only in low-stage PCA; other studies could not confirm this.[176,185] Although not all studies used similar algorithms for calculation of NRF, differences in equations were minor and do not account for the inconsistent value of NRF as a predictive tool in PCA.

All studies that did find NRF useful as a predictive tool, however, contained fewer than 30 patients, whereas studied populations that found it of doubtful value were larger.[176,185] A patient-selection bias might have caused the inconsistent findings concerning NRF and the prognosis of patients with PCA.

Besides NRF, other descriptors of nuclear ellipticity have been tested[175,182] and found useful, but again, all the studies contained small patient groups.

To summarize, karyometric shape analysis resulted in questionable predictive value of histologic material in PCA.[178-181] In FNA material, nuclear shape was not useful in predicting outcome.[182,188,183,184,186] Multiparametric analysis on larger populations with known followup are necessary to investigate the microscopic features and their clinical applicability in prediction.

4.1.1.3 Other morphometric features in prostate adenocarcinoma

Studies using visual grading have shown the importance of nucleolar features for tumor grading.[186-190] Larger nucleolar areas were found in histologic sections of patients who died of prostatic cancer after prostatectomy, than of patients who survived.[191] Among several nuclear and cellular features, such nucleolar features as size and frequency proved most potent in differentiating between cancer and noncancer in FNA biopsies.[175]

4.1.2 Static Cytophotometry

Before a review of the literature on SCM in PCA, it is useful to mention the conclusions obtained from studies of FCM. FCM studies have shown a correlation between DNA pattern, tumor malignancy, and differentiation in prostatic cancer.[192-195] Not all studies confirm the usefulness of measuring DNA content in PCA. Bichel and associates[196] could not find increased aneuploidy in anaplasia and suggested that PCA was a more benign type of cancer. In a FCM study on archival material, Jones and associates[197] concluded that aneuploidy was not required for progression. Ploidy did correlate with grade and stage but not with prognosis, in disaggregated archival material.[198]

SCM studies revealed a correlation among DNA-histogram patterns, the presence of tumor,[117] tumor grade,[199] tumor stage,[109] and survival.[109,200-202] Several studies used FNA material,[109,111,200,201,203] although different DNA-histogram interpretation techniques were used, all except one[111] agree on the value of DNA measurement for clinical diagnosis. Forsslund and Zetterberg[201] stated that, although SCM ploidy levels can accurately discriminate between low- and high-grade malignant tumors, the discrimination depends heavily on the method of measurement and analysis of the DNA histogram.

The DNA malignancy grade (DNA-MG) is a quantitative, reproducible, and objective descriptor of the DNA histogram. Strong correlation with prognosis was found for the DNA-MG in cytologic archival PCA material.[109] An increase in DNA-MG was correlated with a decrease in survival time.[109]

Besides the DNA-MG, division of the DNA histogram obtained with SCM into diploid and aneuploid patterns correlated with prognosis.[109-201] A striking example is the recent study of Forsslund and Zetterberg,[201] who demonstrated the prognostic value of DNA-pattern description with SCM on FNA material. In two extreme patient groups, the optimal limits of the aneuploidy and diploidy definitions were chosen.

When the same criteria were used for DNA description in another population of 79 PCA patients, all patients who died within 5 years of diagnosis had aneuploid DNA histograms, whereas patients who survived more than 5 years had diploid or tetraploid histograms. Greene and associates[128] demonstrated the use of SCM in histologic sections, analyzing ploidy patterns in different foci of PCA. Nondiploidy occurred at lower tumor volume, especially in tumors originating in the peripheral zone, compared with tumors in the transition zone.

To summarize, the results suggest that both FCM and SCM studies agree in showing aneuploid tumors to have more frequent anaplasia and more malignant behavior. However, the clinical application of SCM in the individual patient is not yet clear, partly because of the different definitions of diploidy and aneuploidy used. SCM with histologic slides proved useful in differentiating between PCA of different parts of the prostate.

4.1.3 Texture and Contexture Analysis

The grading according to the method of Mostofi, assessed by three pathologists, correlated well with the number of nuclei per slide, their distance from the glandular center, and nuclear chromatin patterns.[138,204] Petein and associates[177] used nine chromatin texture characteristics of nuclei in tumor imprints and found three descriptors of chromatin clumps (short run-length, long run-length, and contrast) to be significantly different in cancers.

Böcking and associates[175] showed that the irregularity of nuclear arrangement was significantly higher in material from transrectal biopsies of prostatic cancer than in normal prostatic tissue. Architectural features were also useful in discriminating between degrees of differentiation in aspirated material.

It can be concluded that there is evidence that both chromatin pattern and tumor architecture can be quantitated and used in differentiating prostate tumor from nontumor material. Although (con)texture features alone will not be able to grade PCA, multiparametric studies have indicated a role of these features in the prediction of prognosis in PCA in conjunction with other QLM features.

4.2 Transitional Cell Carcinoma (Table 5)

4.2.1 Morphometry

4.2.1.1 Nuclear size

Early studies on the use of QLM in transitional cell carcinoma (TCC) revealed a correlation between nuclear size and tumor grade.[205,206] Nuclear size increased with tumor grade; but, in the same ploidy class, the more differentiated carcinoma cells showed nuclear enlargement.[206] That is a remarkable finding and was explained by a decrease in chromatin concentration in the more differentiated carcinoma. Nuclear enlargement and anisokaryosis proved to be a reliable marker of tumor grades and prognosis in TCC cytologically[207,208] and histologically.[118,1571,188,189,209-214]

Koss and associates[112,215-217] presented several studies on the computer recognition of cells in urinary cytology. Their system included nuclear and cellular size characteristics and several texture features. Whereas the nucleus-to-cytoplasm (N/C) ratio was significantly higher in malignant cells, the nuclear profile area was not. They concluded that computer recognition of benign, malignant, or atypical cells provide important information and offers an approach to automation of cytology of the urinary sediment. Their method was found to be superior to visual grading.[163]

Boon and associates[207,208] could differentiate between grade-1 and 2 cells in voided urine by using the N/C ratio. Although good correlation with tumor grade was shown, the prognostic value of urinary morphometry has not yet been established.

QLM was also used to analyze tumors in histologic sections.[127,143,189,209-211,218] Again, nuclear size correlated positively with tumor grade. Several studies combined FCM and morphometry and compared their prognostic value. The presence of very large nuclei, as measured with morphometry in a study by Blomjous and associates[210] correlated positively with high-grade lesions and could be used to predict tumor recurrence,[210,213] but, was inferior to DNA ploidy as a prognosticator.[210] Considering the high correlation between DNA content and nuclear size, that is not surprising. Helander and Tribukait[218] paid special attention to grade-2 tumors and found FCM and morphometry complementary for recognition of tumor grade. A third group of studies combining FCM and morphometry concluded that morphometric measures were the better predictors of outcome.[157,212] Lipponen and associates[212] did not find the FCM DNA content to be a useful prognosticator and favored only morphometry next to clinical staging. The predictive value of nuclear morphometry was about similar in

these studies, despite the use of completely different selection criteria. It needs to be stated here that the different selection techniques whether based on nuclear size,[169,211] location,[169] or context in cytology,[170,171] seemed to result in similarly good prediction of grade or outcome, this was not the case when a low number (50) of nuclei were measured at random.[171]

Nielsen and associates[214] estimated nuclear volume with point-counting and found that only one of 35 patients with mean nuclear volume below 300 μm^3 died of cancer within a 5-year followup, whereas 18 of 19 patients with a mean nuclear volume above 500 μm^3 developed metastasis or died of cancer. The prognosis of patients with carcinoma in situ could not be predicted from nuclear volume.

Table 5. Morphometry, static cytophotometry, and (con)texture analysis in transitional cell carcinoma.

Morphometry	material (nr)	parameter NRF	ND	NPA	N/C	SDA	NuA	M/V
Levi and associates[205] (1969)	2.(36)		d0					
Fosså and Kaalhus[206] (1976)	2.(25)			g+				
Koss and associates[103,112,215-217]	6.(15)			g0	g+			
Boon and associates[207] (1981)	6.(24)			g+				
Ooms and associates[208] (1982)	6.(41)			g+	g+	g+		
Ooms and associates[169] (1983)	1.(27)			g+				
Helander and associates[143] (1984)	4.(27)	g+		g+				
Helander and associates[209] (1985)	4.(28)			g+				
Montironi and associates[127] (1985)	1.(35)	g+		g+				
de Sanctis and associates[213] (1987)	1.(30)			p+				
van der Poel and associates[170] (1988)	6.(50)			g+				
Helander and Tribukait[218] (1988)	2.(125)			d+				
Blomjous and associates[210] (1989a)	1.(80)			p+				
Blomjous and associates[211] (1989b)	1.(61)			g+				
Blomjous and associates[188] (1990)	1.(61)			g+/s+/p+				
Lipponen and associates[212] (1990a)	1.(83)			p+		p+		p+
Lipponen and associates[157] (1990b)	1.(30)			g+				
de Prez and associates[116] (1990)	3.(46)			g+				
van der Poel and associates[122] (1991)	4.(28)	g0		g+		g+	g+	
van der Poel and associates[208] (1992)	1.(36)		p+	p+				
Borland and associates[267] (1993)	1.(14)		p+	p+				
Densitometry		ploidy		bad prognosis/highgrade				
Levi and associates[205] (1969)	2.(36)	g+						
Lederer and associates[232] (1972)	3.(21)	g+						
Fosså and associates[234] (1975)	2.(24)	g+		triploid/hexaploid				
Atkin and associates[236] (1979)	2.(61)	0						
Hofstädter and associates[115] (1980)	3.(49)	p+		not in individual case				
Bjelkenkrantz and associates[231] (1982)	1.(3)	g+						
Montironi and associates[127] (1985)	1.(27)	g+		tetraploidy/aneuploidy				
Bass and associates[126] (1987)	6.(67)	g+						
Koss and associates[112] (1987)	6.(54)	g+						
Stöckle and associates[114] (1987)	2.(46)	p+		high 5cER				
de Prez and associates[116] (1990)	3.(46)	g+		high PI and PBI				
van der Poel and associates[122] (1991)	4.(28)	g+		high 5cER and 2cDI				
(Con)texture analysis		ChP		Contexture				
Fosså and Kaalhus[206] (1976)	2.(25)	g+						
Koss and associates[103,112,215-217]	6.(15)	g+						
Helander and associates[143] (1984)	4.(27)			g+ (nuclear volume densities)				
de Prez and associates[116] (1990)	3.(46)	g+ (run length features)						
van der Poel and associates[122] (1991)	4.(28)	g+ (Markovian features)						

4.2.1.2 Nuclear shape

In contrast with its use in prostatic adenocarcinoma, few studies discuss the use of nuclear or cellular shape descriptors in TCC.[184,266,267] From cytologic criteria, we know that cells in lower-grade tumors have more elongated nuclei, whereas cells in high-grade tumors contain nearly circular or irregularly-shaped nuclei. NRF[178] is less suitable for discrimination between these two types of shape,[96] but was found useful to differentiate tumor grade.[127] The use of histologic material in which elongated nuclei could be cut in the tangential plane and appear more round might have caused the relatively low NRF values in low-grade tumors in the study cited.[127] As discussed earlier, NRF is not preferable as a descriptor of nuclear shape.[96] Although other studies used nuclear shape in multiparametric analyses of urinary cytology[184,219] or tumor imprints,[116] important prognostic value has never been found.

4.2.1.3 Other morphometric features

Besides nuclear size and its standard deviation, the N/C ratio[207,208] and the nucleolar-to-nuclear ratio[221] positively correlated with tumor grade. Cytoplasm is much more subject to degradation or deformation than the nucleus, especially in the Feulgen staining, so material processing technique will strongly influence cytomorphometric analysis. Hence, cytoplasmic morphometry is less suitable as a routine diagnostic procedure. Finally, a group of multiparametric studies needs attention. These studies described the recognition of atypical and benign cells in voided urine,[55,112,163,184,209,215,217,219] imprints,[116] or histologic material.[122] The early findings of Koss and associates[217] were elaborated on[163] and resulted in the designation of the "selective mapping algorithm",[184] in which "objects" were selected for higher-magnification analysis. Cells in the urinary sediment were scored as benign, atypical, suspicious, or malignant and that rendered the system useful for automated prescreening of slides. However, as Brugal and associates[219] stated: "Although the rationale involved in the hierarchic decision tree would have to be based on statistical approaches, ... the lack of confidence in the cytologist's cell-to-cell reference classification would alter the meaning of any sophisticated cell-to-cell automated decision making based on it." Clinical application of such a system is not yet feasible, even apart from the costs of the fully automatic cell-scanning device.

It can be concluded that nuclear size and its standard deviation, measured with either point-counting, cytomorphometry or histomorphometry showed significant increase with tumor grade and seemed to have prognostic value.[211,214] However,

absolute values for nuclear size differ between studies, because of preparation methods, and standardization of these methods is mandatory. Irregularity of nuclear shape was correlated with higher grade, but its predictive value has not been unequivocally proved. So far quantitative features seem to be most predictive of grade and outcome. Tumor stage and QLM, where investigated, showed less correlation.

4.2.2 Static Cytophotometry

Tribukait and associates[194,220,222] found, in FCM studies, a correlation among DNA ploidy patterns, proliferative activity, histologic classification, and prognosis. Aneuploid tumors had a strong propensity to progress into invasive and metastatic lesions. Tumors with a tetraploid DNA pattern, however, appeared to have a more moderate potential for malignancy. Heterogeneity was often found in the primary tumor[223,224] and between the primary tumor and the metastasis.[225] The use of FCM in the followup of patients seemed rewarding in several studies.[102,210,221,226-230]

Like FCM, histologic visual classification correlated with the ploidy pattern as assessed by SCM in both sectioned material[115,127,128] and smears.[118,126,231,233] Determination of individual prognosis of low-malignancy tumors and differentiation of histologically borderline cases, however, were not possible.[115] In general, it was concluded that low-grade TCC had diploid cell populations, whereas more malignant tumors showed tetraploid, polyploid, or triploid DNA patterns with increasing tumor grade.[118,205,234,235]

Studies correlating SCM data from TCC with prognosis are rare. Stöckle and associates[235] found DNA ploidy, proliferation, and number of cells with more than 5c DNA content to correlate with survival in disaggregated, deparaffinized archival material. Urinary cytology material, well suitable for SCM DNA analysis, was also successfully used in several studies and proved to offer information in addition to that gained from visual interpretation.[112,126]

Like FCM, SCM analysis revealed DNA heterogeneity in bladder tumors.[205,236] More malignant behavior was found in tumors with a higher number of cellpopulations.

To summarize, it is clear that aneuploid tumors behave more malignantly than diploid tumors. Some studies have shown that aneuploidy does not necessarily correlate with malignancy.[225,237] Furthermore, several descriptors of the DNA histogram indicate more heterogeneous cell population in more aggressive tumors. DNA content alone, however, has not been shown to be of clinical use in assessing

tumor prognosis. In contrast, data from individual followup and measurement of treatment response can be applied in combination with other karyometric or clinical features. In particular minimally invasive techniques, such as bladder washings can offer a rich harvest of cells suitable for SCM analysis.

4.2.3 Texture and Contexture Analysis

Fosså and Kaalhus[208] found the less-differentiated tumors in a given ploidy group to have greater chromatin concentration. Several texture features changed significantly with grade[116,122] patterns were more coarse and irregular in high-grade tumors. However, the absence of uniformity in the features used, renders comparison of data difficult.

4.3 RENAL CELL CARCINOMA (Table 6)

4.3.1 Morphometry

4.3.1.1 Nuclear size

In contrast with prostatic adenocarcinoma and transitional-cell carcinoma, renal-cell carcinoma (RCC) comprises several different types of cells. Skinner and associates[72] differentiated clear-cell, granular-cell, and spindle-cell RCC. Metastatic rate[63] and survival[72] were found to vary for the three types of tumor. However, heterogeneity of DNA or cell type often occurs in RCC, so morphometric approaches to nuclei, regardless of cell type or heterogeneity, might not yield an appropriate selection of nuclei.[238-243]

The presence of large nuclei correlated with a poor prognosis in several studies.[238-240,243] Nuclei from metastatic RCC were found to be larger and more polymorph than those in the primary tumor,[246] but the patient groups studied were small. Although correlation of nuclear size and survival[238,240] existed, Tosi and associates[238] found 18% (n=17) false positives in stage-1 RCC. Moreover, Bibbo and associates[240] did not find the increase in nuclear size significantly different between patients free of disease and those who died from the disease during an 11-year followup. Both studies used histologic material. Morphometry applied to tumor imprints[242] confirmed the correlation of nuclear size and tumor grade.

Table 6. Morphometry, static cytophotometry, and (con) texture analysis in renal cell carcinoma.

	material (nr)	parameter NRF	NPA	NuA	Npo	Ell	Fell
Morphometry							
Colvin and Dickerson[238] (1978)	1.		p+				
Gilchrist and associates[243] (1984)	1.(32)		p+ ("discordant nuclei")				
Tosi and associates[239] (1986)	1.(67)	p0	p+ (> 32 μm^2)			p+	
Bibbo and associates[240] (1987)	1.(19)	p0/g+	p+/g+			p+/g+	
Murphy and associates[241] (1990)	1.(20)	p0	p0				p+
vanden Houte and associates[242] (1991)	3.(16)		g+				
van der Poel and associates[244] (1993)	1.(121)		p+	p+	features describing heterogeneity		
Densitometry							
		ploidy		bad prognosis/high grade			
Bennington and associates[123] (1983)	1.(21)	p+		aneuploidy			
Ljungberg and associates[124] (1986a)	1.(55)	p+/s+/g+		increased DNA content			
Ljungberg and associates[254] (1986b)	1.+2.(32)	p+		aneuploid metastasis			
Bibbo and associates[240] (1987)	1.(19)	p0/g0					
Stöckle and associates[233] (1990)	2.(49)	p+		hypertiploidy			
vandenHoute and associates[242] (1991)	3.(16)	g+		DNA-ploidy/heterogeneity			
(Con)texture							
		ChP		Contexture PND NCr			
Bibbo and associates[240] (1987)	1.(19)			p0/g0 p+/g+			
vanden Houte and associates[242] (1991)	3.(16)	g+					
Mulders and associates[245] (submitted)	1.(52) $T_{1,2,3}N_0M_0$	p+					

4.3.1.2 Nuclear shape

Murphy and associates[241] measured 25 shape descriptors, earlier found to be of possible predictive value in prostatic cancer,[182] in 10 patients who survived 5 years after surgery for RCC and 10 patients who died of metastatic disease. Two individual shape descriptors (the relative mean of the 10 largest convexity values and median quartiles of ellipticity according to Fourier analysis) successfully classified the prognosis of 18 of the 20 patients. Ellipticity value increased as contours deviated from an ellipse. The more simple elongation factor with prognostic value, as established by others,[239] was not measured in the study. Murphy and associates[241] found higher ellipticity values in the nonsurvivor group; that suggested an unfavorable prognosis when the nuclear shapes deviated from elliptical, which does not necessarily contradict earlier findings by Bibbo and associates.[240] They also found a strong correlation between nuclear elongation and survival. Murphy and associates[241] could classify 16 of the 20 patients correctly, using NRF, its values being higher in the nonsurvivor group; which does not agree with Bibbo and associates.[240] Bibbo found NRF to correlate with tumor grade, but not with survival.[240] In addition, Tosi and associates[239] found neither nuclear elongation nor NRF of prognostic value in stage 1 RCC. The discrepancy in findings of the predictive value of nuclear shape in RCC resemble that in the findings in prostatic adenocarcinoma, and small patient groups most likely again caused the inconsistent findings. Moreover, the tremendous tumor heterogeneity, often present in RCC may have hampered analysis of the most significant area in the tumor. In a recent study we found several nuclear features to correlate with survival[244] and progression.[245] Among them nuclear shape, as described by features derived from the Freeman chain code of the nuclear contour. However, in a multivariate analysis features that described tumor heterogeneity showed strongest correlation with tumor behaviour among the karyometric features. For locally confined disease ($T_{1,2,3}N_0M_0$) heterogeneity in chromatin pattern was even the strongest predictor of tumor progression.[245]

To summarize the results of the morphometric studies on RCC, it is clear that both size and shape of tumor-cell nuclei provide some, but not much information about prognosis. Although Tosi and associates[239] found a rather clear cutoff value for nuclear profile area between survivors and nonsurvivors, others merely found an increase in nuclear profile area with grade and tumor-related death.[238,240,243] The reports on shape analysis are too ambiguous to support any consistent conclusion. Although nuclear size and shape were successfully used for visual grading of RCC,

studies on larger patient groups are needed to test the predictive value of quantitation of these features as prognosticators in RCC. Tumor heterogeneity will be an important phenomenon in further analyses.

4.3.2 Static Cytophotometry

FCM studies[247,252] indicated the usefulness of tumor ploidy in the prediction of outcome, metastasis[251,252] and grade[241,248,251,252] of both fresh[247,251] and archival[252] material of RCC. However, results are not always consistent, and the routine application of FCM in RCC is doubtful.[253]

Like FCM data, SCM data on the predictive value of DNA content are not always unanimous.[123,124,240] Several studies used SCM to examine sections[123,124,254,255] and smear material[231,256] in RCC.

Ljungberg and associates[124] found diploid or near-diploid DNA histograms in 32 of 33 patients still alive 10 years after surgery for RCC. All 22 patients who died within 4 years after surgery showed nondiploid DNA histograms. In some patients whose clinical course deviated from the expected in clinical grade and stage, survival correlated well with the SCM DNA pattern. However, the good correlation between DNA content of the primary tumor and survival was lost in metastasized RCC.[254] Patients with diploid and neardiploid metastases had significantly longer survival than those with aneuploid metastases, whereas the correlation between ploidy pattern of the primary tumor and survival was lost.[240,254]

SCM of nonsectioned material is rare. Some correlation of survival with DNA content and of heterogeneity with tumor grade[242] and survival[233] was found, but, patient groups were small[242] or seemed nonrepresentative.[233]

To summarize the results of SCM studies in RCC, it can be concluded that RCC show a considerable DNA heterogeneity.[233,255] In some studies, DNA content correlated well with tumor grade. Correlation with prognosis is less clear, perhaps because metastases occur in diploid and aneuploid tumors at similar frequency. However, aneuploid metastases show a more progressive growth; therefore, the DNA content of metastases correlated well with prognosis.[226] The divergence in DNA-ploidy pattern between the primary tumor and the metastases is often high.[225]

4.3.3 Texture and Contexture Analysis

Bibbo and associates[240] found an increase in nuclear crowding[138] in the tumors with worse prognosis. The proximate nuclear distance (see Glossary) did not have any

predictive value. Because nuclear crowding (see Glossary) yields a rough estimate of the N/C ratio, that means an increase in N/C ratio in more malignant tumors, as was found for transitional-cell carcinoma. Nuclear crowding can easily be incorporated into QLM routines and should certainly be tested on larger patient populations.

5 CONCLUSIONS

The use of QLM in urologic oncology has been explored in many ways. This paper has reviewed the application of QLM in the diagnosis of prostatic adenocarcinoma, transitional-cell carcinoma, and renal-cell carcinoma.

Karyometric analysis of prostatic adenocarcinoma (PCA) revealed an increase in nuclear size and anisokaryosis in more malignant tumors. Nuclear shape analysis as a predictive tool is questionable for PCA and not yet suitable for routine application, in light of the inconsistency of findings which is most likely due to patient selection bias. DNA content in PCA, measured with either SCM or FCM showed more abnormal patterns in both histologic and FNA material in tumors with a worse prognosis, that seemed to be important merely from a research point of view, rather than as a tool for individual clinical tumor grading. Tissue-architecture descriptors, such as glandular size, shape, cellularity, and descriptors of chromatin pattern correlated well with routine visually assessed PCA tumor grade, in both histologic and cytologic material. Additional information obtained with these features could give insight into glandular formation as a measure of tumor differentiation as applied in the visual grading.

Morphometry of transitional cell carcinoma (TCC) showed an increase in nuclear profile area with tumor grade, stage, and prognosis. The predictive value of shape descriptors in TCC was evaluated in few studies, but correlation with tumor grade has been reported. Absolute values of both nuclear size and shape features, however, vary among the different studies. SCM data agree with FCM findings: the presence of aneuploid populations correlated with high tumor grade and worse prognosis. Description of chromatin and nuclear patterns enabled assessment of tumor grade in TCC. However, correlation with prognosis has not yet been established. Because TCC show a recurrent and multifocal incidence, recurrent tumors and random biopsies of tissue next to the tumor need further investigation. Recent studies showed no prognostic value of nuclear size in carcinoma in situ of the bladder, but further study of the different growth patterns of TCC and its precursors, such as

atypia, is necessary. Moreover, longitudinal quantitative follow up be means of karyometric analysis of urine cytology enables comparison of subsequent samples and could be a tool for early detection of tumor recurrences or progression.

Of the three urologic tumors discussed, the least literature is available on renal-cell carcinoma and QLM. An increase in nuclear profile area correlated with a worse prognosis. Of the shape factors, descriptors of ellipticity seemed to be important. Not all studies agree on the value of DNA assessment in RCC regarding primary tumor heterogeneity; aneuploidy of tumor metastasis was found to be a more important prognostic factor. Of the textural features, nuclear crowding in the tumor was found to correlate with prognosis.

DNA analysis suggested that aneuploidy correlates with greater malignancy in the three urologic tumors. Aneuploidy has been classified into tetraploidy, triploidy, and hyperdiploidy. Tetraploid cells appear in the more benign tumors with polyploid cells. Triploid populations seem to merge from the tetraploid cell lines because of loss of chromosomal material. Hyperdiploid aneuploidy, as a result of gain of chromosomal material of a diploid population, possibly predicts malignancy. Partly because of tumor heterogeneity, there are too many exceptions to the rule that: "diploidy equals non-malignant behavior" for DNA analysis alone to be a strong predictive factor.

The reason for applying QLM, as discussed in the introduction, was the high inconsistency of visual tumor grading. The results of QLM studies so far, however, indicate that it is a more consistent, way of tumor grading. Three types of inconsistency, however, still exist:

- The heterogeneity of tumors renders results largely dependent on the method of selection of material used for analysis. Multiple samples and clear selection criteria are necessary to overcome these problems.
- Preparation method influences results and standardization of these techniques is mandatory.
- Differences in methods of quantitation, consisting of differences in image recording and processing techniques and nonuniformity of features used warrant a clear description of apparatus and software. Because several studies agree on various characteristics as prognostic factors in urologic oncology, uniform prospective studies using these features are to be performed.

So far, quantitative pathology is valuable in support of pathologic diagnosis only in research settings. As long as standardized automated fixation, embedding, staining,

selection, and measuring techniques are lacking, data obtained with various preparation methods will differ too much to support consistent conclusions. Therefore QLM is not yet applicable as a routine tool for tumor grading, but can play an important role in patient followup because of the possibility of qualitative comparison of subsequent samples, such as obtained in urinary cytology.

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Chapter 1

KARYOMETRIC ANALYSIS FOR THE GRADING OF UROTHELIAL CELL CARCINOMA OF THE BLADDER

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INTRODUCTION

As discussed in the introduction of this thesis, quantitative analysis of light microscopic images provides objective grading. In this chapter our studies on the grading of transitional cell carcinoma of the bladder are discussed. Earlier studies on the quantitative grading of transitional cell carcinoma (TCC) showed good correlation between karyometric grading and histopathological interpretation by the pathologist.[1] In the present studies we evaluated the use of an image analysis system as used by Ooms et al. (1984)[1] for quantitative grading of cytological and histological material of bladder cancer patients. Since preparation technique plays an important role in the nuclear appearance,[2] several embedding and staining methods were applied to the cytological material. The Kontron system, used in the study in the first part of this chapter enabled analysis of only a limited number of nuclei and nuclear features. We evaluated the role of nuclear profile area, analyzed interactively with the Kontron system for the grading of cytological material.[3] Visual grading of tumors by microscopic images, however, is based on an extensive number of tissue, cellular, and cell nuclear characteristics.[4] Hence, in a second study multiple nuclear features were studied for the grading of histological material from bladder cancer using the IBAS 2000 system.[5] Earlier studies[6] reported successful application of ultra-thin (2 μm) sections using plastic-embedding techniques. Since this type of section allows stereological analysis of data and estimation of three-dimensional structures, plastic embedding instead of routine paraffin embedding techniques were applied.

The aims of the studies presented in this chapter are: 1. To evaluate the correlation of nuclear size in cytological material with histological tumor grade and nuclear size; 2. Comparison of different cytological preparatory techniques and their influence on nuclear size; 3. To investigate the karyometric features useful for the grading of bladder tumors in a multivariate analysis. In Chapter 3 and 4 we present clinically oriented studies on the applicability of karyometric grading.

MATERIAL AND METHODS

Material for analysis with MOP-video plan

Cytological material

The material for cytomorphometrical analysis consisted of 50 consecutive cases, 39

with the histological diagnosis of bladder tumor and 11 with inflammatory changes in biopsy material. Each bladder tumor was graded histologically according to the WHO system by one pathologist. Eleven were graded as grade 1, 16 as grade 2, and 12 as grade 3. In all patients the follow up was complete until 2 years after the bladder washings. Prior cytology and histology specimen were available.

For the cytological preparations the bladder was rinsed with saline solution .[7,8,9] The bladder washings were obtained in the Westeinde Ziekenhuis in The Hague during 11 months (february-december 1986). After rinsing, the sample was centrifuged and the cell pellet resuspended in 50 cc fixation fluid prior to further processing. The fixative used was composed as follows: 70cc polyethylene glycol (MW 300), 800cc ethanol 96%, and 130cc distilled water (Kryofix, Merck). After centrifuging, the sediment was divided into two parts. One was used to make the smears, the other to embed in paraffin. In this latter case the pellet was dehydrated in ethanol 96% twice, ethanol 100% twice, and toluene once. Paraffin was added to the dehydrated pellet and after it was solidified the test tube was broken, and sections were prepared from the block. Because not every sample contained enough material, not all techniques could be used in all cases. One smear and section were stained according to the Papanicolaou method (Pap-smear and Pap-section) and according to the Feulgen method (Feulgen smear and Feulgen section).

MOP video plan system (KONTRON)

The cytomorphometrical measurements were made by means of a microscope connected with a computer MOP-video plan (KONTRON, Zeiss, Germany). This system consisted of a digitizer tablet and a video screen. The microscopic image was recorded and projected on the screen. With a mouse the nuclei were manually delineated. 50 nuclei per slide were delineated and measured for nuclear profile area by the computer. This procedure took approximately 15 minutes per slide. All measurements were done by one person not knowing the histological grading.

Selection of nuclei for cytomorphometry

To select nuclei for analysis a stepwise screening of the slide was conducted prior to analysis. The slide to be measured was first screened to obtain a general impression and to circle the diagnostic cell groupings of the highest grade (for definition see further text). Only those nuclei that were clearly visible and not covered by other nuclei were taken for measurement. Three types of diagnostic cell groups were

distinguished. Type 1: Large, dense papillary groups with smooth outlines. Type 2: smaller, less dense papillary groups with regular outlines. Type 3: Loose clusters of a few clearly malignant cells. In addition, the washings contained sheets with ragged borders and lacking a papillary architecture.

Grade-1 tumor cells are almost exclusively in type-1 cell groupings (Figure 1). The nuclei are often pale in the Papanicolaou staining with nuclear clearing and there is a prominent nuclear envelope and clumping of some chromatin. The nuclei are predominantly oval or round some with abnormal shapes. The nuclei measured were those situated at the edges of the papillary groups. The nuclei in the center are not clearly visible, thus do not fit for analysis. Oval as well as round nuclei were measured.

Grade-2 tumor cells are arranged in predominantly type-2 cell groupings and sometimes as single cells or in type-1 groupings (Figure 2). Cases with inflammatory reaction in histological evaluation have mainly isolated cells and sheets. The cells are often cylindrical with an eccentric nucleus. In this group there are no papillary groups so nuclei in single cells are measured.

Grade-3 tumor cells area mainly arranged as single cells and in addition in type-3 cell groupings (Figure 3). Cells selected for measurement have a very high N/C ratio[10,11] and the chromatin pattern shows uneven distribution. Often there is more than one, irregularly shaped large nucleolus.

Prior to measurement the slide was screened and the diagnostic cell groupings of the highest malignancy grade were circled for analysis. Thus, in case of an admixture of type-1 cell groupings (with no or little atypia) and type-3 cell groupings (clearly malignant), the latter were circled for measurement. In case of type-2 and type-3 cell groupings, the scattered single clearly malignant cells in the neighborhood of the cell groupings were also analyzed.

For comparison with histomorphometric evaluation, histological material was obtained by transurethral resection after the bladder washing. Each biopsy was processed into 3 blocks. The grade-1 tumors were all noninvasive in contrast to the grade-3 tumors. In each case the most abnormal parts of the tumor tissue were selected for histomorphometric analysis. In these areas, three types of cells were measured: A, deep cells (DEEP); cells near the stroma, B, superficial cells (SUPERF); cells near the bladder lumen, C, cells with large nuclei (LARGE).[1] In each tumor 240 nuclei were analyzed. Cells with evident pyknotic nuclei or degenerating cells were not selected for this study.

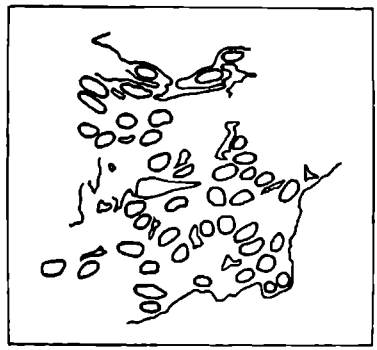


Figure 1. Example of camera-lucida drawing of grade-1 tumor cells.

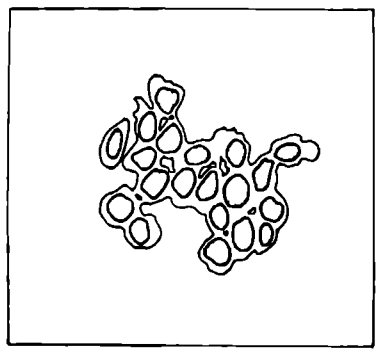


Figure 2. Example of camera-lucida drawing of grade-2 tumor cells.

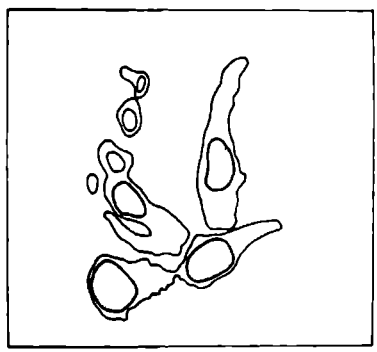


Figure 3. Example of camera-lucida drawing of grade-3 tumor cells.

Material for analysis on IBAS 2000 system

Histological material

The material consisted of 28 cases with the diagnosis of bladder tumor, graded histologically according to the WHO system. The histological material was obtained by transurethral resection and consisted of 9 grade-1, 11 grade-2, and 8 grade-3 tumors. The material was fixed in an on ethanol-based coagulant fixative as described above (Kryofix, Merck).

Plastic embedding

The histological material for analysis with the IBAS 2000 system was embedded in plastic. Glycolmethacrylate (GMA) was used in the process of the plastic embedding (Table 1).

Table 1. Mixtures used for plastic-embedding

Solution A	
Glycol methacrylate with 200 ppm hydroquinon (Merck-Schuchardt; no. 800588)	90 cc.
2-butoxyethanol (Merck-Schuchardt; no. 801554)	10 cc.
Benzoylperoxide (with 20% H_2O) (Baker; no. 9104)	0.5 g%
Solution B	
Polyethylene glycol 400 (Fluka AG; no. 39430)	15 parts
N,N-dimethylaniline (Fluka AG; no. 39455)	1 part

The histologic material was incubated in solution A (Table 1) overnight. After incubation solution A was decanted. For polymerization, a mixture of 30 parts of solution A and 1 part of solution B (Table 1) was prepared, stirred by hand for two minutes, and added to the material. The histologic material was placed into a plastic moulder. A block holder was placed on top and covered with paraffin. The plastic was ready for cutting after two hours, 2 μm sections were made from the blocks. The GMA sections stretched well on a water-bath surface. At 100x magnification the sharp-depth was 1-2 μm and approached the section thickness. This enhanced the chromatin-pattern analysis and nuclear-boundary tracing

IBAS 2000 system

Image analysis was performed by means of a microscope (Axioplan, Zeiss) connected with a dual processor system, the IBAS 2000 (Image Basis Analysis System) of Kontron Electronics, Zeiss.[12,41] The marked areas were scanned with a 100x oil immersion objective by a Hitachi CCTV video camera. At this magnification the pixel size was 0.012 μm^2.

The menu based image processing available in the system was used for morphometrical and densitometrical measurements. The SCRAPE function was used to delete objects less than 10 μm^2, since our earlier study[3] revealed no smaller urothelial-cell nuclei.

For the IBAS 2000 there was no commercially available software to calculate the texture features. For this reason software was developed in TURBO-Pascal to recognize the nuclei and perform the Markovian texture analysis on an IBM compatible PC. The selection of tumor areas in the histological sections was done by the pathologist in the haematoxylin-azofloxin-stained (HA) sections. The section adjacent to the HA-section was Feulgen stained and used for the karyometric analysis. In each Feulgen-stained slide 200 nuclei in the marked areas were measured. This took about 20 minutes per side. Since the IBAS 2000 system segments nuclei semi-automatically we did not apply the selection criteria developed by Ooms et al. (1983)[1] and used in the comparison with cytomorphometry earlier in this chapter. Randomly selected nuclei from all two (DEEP, SUPERF) tumor areas were taken.

Table 2. Morphometric and densitometric karyometry features tested in multivariate analysis.

Morphometry

1. real mean AREA (rAREA) = mean (mAREA) - (10/3)
2. PERIMETER
3. percentage of nuclei over 60 μm^2 AREA: 60 μm^2 ER
4. percentage of nuclei over 90 μm^2 AREA: 90 μm^2 ER
5. FormELL: $\frac{\text{minor axis}}{\text{major axis}}$
6. FormPE: $\frac{4\pi * \text{AREA}}{\text{PERIMETER}^2}$

Features derived from the smoothed Freeman difference chain (SFDC) code[67].

7. MAC (Mean Absolute Curvature)

$$= \frac{1}{C * N} \sum_{i=1}^{N} K(n)$$

(C = number of contour pixels
N = width of smoothing operator
K(n) = SFD-value in *n*)

8. MBEN (Max. Bending Energy) (= difference between highest and lowest value in SFDC-code)

9. PASS (= number of passes through threshold in SFDC-code)

Densitometry

10. optical density (OD): mean optical density of pixels in nuclear boundary: $-\log_{10}$ TRANSMISSION
11. integrated optical density (IOD): OD * AREA
12. 2c Deviation Index (2cDI):[17]

$$2cDI = \frac{1}{N} \sum_{i=1}^{N} (c_i - 2c)$$

13. 5c Exceeding Rate (5cER): the percentage of nuclei per slide over 5c DNA value minus the exponents of 2c.[17]
14. mean total DNA content: mtDNA: see equation (5)

Nuclear features and stereology

The computer calculated the features AREA, PERIMETER, FormELL, and FormPE (Table 2) of the Feulgen-stained slides. The mean value of AREA was not equal to the mean value of the maximum profile area.

The necessary corrections were well understood, and described in the literature.[13] Two corrections were used in the current study. First, as an acceptance criterium for nuclei AREA was required to be larger than 10 μm^2. This value is referred to as cut-off area (s_{cutoff}). Small caps of nuclei therefore were not taken into account. For plane sections of spherical nuclei an exact correction term could be calculated,

$$<s_{profile}> = <\text{AREA}> - (s_{cutoff}/3), \qquad (1)$$

where the angular brackets denote mean values of all nuclei (cf. Eqs. (12.6) and (12.12) of Kok[14]). Eq. (1) was used to correct for the effect of the lower detection limit on the profile size. Second, one should correct for the fact that not all nuclei were cut centrally (for example for spherical nuclei the centre of the sphere was often not contained in the cut section, especially for thin cut sections). Hence, the nuclear profile $s_{profile}$ obtained was not the largest possible profile area, that could have been obtained by cutting somewhat higher or lower. In the model situation that all nuclei are spherical the correction formulas can easily be worked out.

Stereology

From the measured morphometrical features for the two-dimensional microscopical image the mean value of an essentially three-dimensional feature of the nuclei, namely the volume, could be derived. Matsushita[15] used the following approach to this problem. From the mean profile area $<s_{profile}>$ he calculated a length, $d_n = 2\sqrt{(<s_{profile}>/\pi)}$, referred to d_n as mean nuclear profile feature, then used Eq. (5.25) of Weibel[13], $D_n = (4d_n/\pi) - t$ to define a 'mean nuclear diameter' D_n. As a last step he used $V_n = (\pi D_n^3)/6)$ to calculate what he referred to as nuclear volume V_n. In the present study the feature t was used, in such a way that $2t$ is the section thickness.

In the current approach a slightly different and more precise formula (cf. Eqs. (31.3) and (50.3) of Kok[14]) was used, $<V_{nucleus}> = <\text{height}> * <s_{profile}>$, which holds for spheres and ellipsoids of revolution. For spherical nuclei one can derive for very thin (i.e., $t=0$) sections (Ref. 25, Eq. (72.2))

$$\langle V_{nucleus} \rangle = \pi^2 \frac{\langle r^2 \rangle}{\langle r^{-1} \rangle} \qquad (2)$$

For more general nuclear shapes we therefore shall use the formula

$$\langle V_{nucleus} \rangle = \pi^{1/2} \frac{\langle s_{profile} \rangle}{\langle s_{profile}^{-1/2} \rangle} \qquad (3)$$

which reduced to (2) for spherical nuclei, indeed. For sections with $2t > 0$ correction factors could be calculated for both the numerator and the denominator of the right-hand side of (2). The following first-order correction was used, by which the right-hand side of (3) must be multiplied[25], so that

$$\text{mtVOLUME} = \pi^{1/2} \frac{\langle s_{profile} \rangle}{\langle s_{profile}^{-1/2} \rangle} \times \left(1 - \frac{4 \langle (s_{profile})^{1/2} \rangle t}{\pi^{1/2} \langle s_{profile} \rangle} \right) \left(1 + \frac{\pi^{1/2} \langle s_{profile}^{-1} \rangle t}{2 \langle s_{profile}^{-1/2} \rangle} \right) \quad (4)$$

Densitometry

Densitometric features consisted of optical density (OD) and integrated optical density (IOD) (see Table 2). Nuclei of white blood cells, almost always present in the slide, were used as reference using the correction factor of 1.19 for the difference in hydrolysis properties.[16] The algorithms according to Böcking et al.[17] were used to describe the DNA histogram. The 5c Exceeding Rate (5cER) and the 2c Deviation Index are defined in Table 2.

To calculate the mean total DNA content per nucleus we used the formula

$$\text{mtDNA} = \frac{\langle V_{nucleus} \rangle}{\langle V_{slice} \rangle} * \text{IOD.} \qquad (5)$$

The average volume of the cut slice of the nucleus in lowest-order approximation in the section-thickness parameter t clearly is $\langle V_{slice} \rangle = 2t \langle s_{profile} \rangle$. From (5) one finds to next order of approximation in t (using Eqs. (72.9-10) a simple form for mtDNA:

$$\text{mtDNA} = \frac{4/(\pi(\pi)^{1/2}) \langle (s_{profile})^{1/2} \rangle + t}{t} * \text{IOD.} \qquad (6)$$

Texture analysis

Texture features used were thoroughly discussed in earlier studies.[18,19,20] Five (described in Table 3) of the 21 Markovian features were used in biological images, because a high correlation has been found among subsets of these 21 features.[18,19]

The Markovian analysis describes 'stickiness' of pixels with the same grey value. The equations use grey-value classes for comparison to neighboring pixels. To fill these classes a method of requantization was necessary: histogram equalization (H) and linear requantization (L) were used in this study for each nucleus.[20] H1 described the entropy of the co-occurrence matrix. The coarser the chromatin pattern, the greater will be the tendency of the high values to concentrate near the diagonal of the matrix, this was expressed by H3. H2 expressed the tendency of the neighboring pixels to be of different grey value. H4 and H5 measured whether the grey values were evenly distributed over the image.[20] L1 and L5 were the same features in the linear requantization method.

Table 3. Texture features.

selected Markovian texture features:

16. H1+L1 ENTROPY:

$$H1 = \sum_{i=1}^{R} \sum_{j=1}^{R} -t(i,j) \log t(i,j) \quad \text{for } t(i,j) <> 0$$

17. H2+L2 DIFFERENCE MOMENT:

$$H2 = \sum_{i=1}^{R} \sum_{j=1}^{R} (i-j)^2 \, t(i,j)$$

18. H3+L3 INVERSE DIFFERENCE MOMENT:

$$H3 = \sum_{i=1}^{R} \sum_{j=1}^{R} \frac{t(i,j)}{1+(i-j)^2}$$

19. H4+L4 ROTATION MOMENT:

$$H4 = \sum_{i=1}^{R} \sum_{j=1}^{R} ((i-\bar{t})^2 + (\bar{j}-t)^2) \, t(i,j)$$

with $t = R/2$ for histogram equalization

and $t = f_{m,normal}$ for linear requantization

20. H5+L5 INVERSE ROTATION MOMENT:

$$H5 = \sum_{i=1}^{R} \sum_{j=1}^{R} \frac{t(i,j)}{1+(i-t)^2 + (j-t)^2}$$

R (here R = 8) denotes the number of grey values in the requantized grey value scale, and t(i,j) the frequency of transition of a grey value i followed by value j. $f_{m,normal}$ denotes the median grey value of nuclei of normal specimens.[20,51]

Feulgen staining

For both studies similar reagents were applied for staining according to Feulgen[19]. For the Feulgen staining of the plastic sections, we tested different times for hydrolysis in 5 N and 1 N HCl and the Schiff's reagent (Merck 9033). Eventually, the staining method according to Feulgen was selected with hydrolysis in 5 N HCL for 30 minutes at 21°C and 30 minutes in Schiff's reagent at the same temperature. The Feulgen-stained sections were used for the IBAS measurements, for visual grading and marking of parts of the tumor to be measured, haematoxylin-azofloxin-stained sections were prepared. The marks were copied on the parallel Feulgen-stained sections.

Statistical analysis cytomorphometric analysis

In the univariate cytomorphometrical analysis we choose as a tool for data reduction the calculation of the natural logarithm of the mean nuclear area value in order to obtain a variance stabilizing transformation. The regression of grade (low versus high) on the histomorphometrical and cytomorphometrical variables to be considered was studied using a probit model.[21] To test whether the cytomorphometric variables can be used in addition to histomorphometric variables in the grading of the bladder tumors and vice versa, the likelihood ratio test was applied.[22] The test was used to investigate whether one cytopreparatory technique is sufficient. The statistical package GLIM[21] was used to perform the analysis for the probit model.

In the multiparameter analysis using the IBAS 2000 system discriminant analysis for karyometric tumor grading was performed using the SPSS/PC+ statistical package. For entry in the discriminant analysis the method according to Wilks was used.[18] The F to enter is set one.

RESULTS CYTOMORPHOMETRIC ANALYSIS

In Table 4 the mean nuclear profile area values of the variables (log mean values and SD values) of the four groups are shown. For the bladder carcinomas, all histomorphometric and cytomorphometric values increase with increasing grade.

Table 4. The mean logarithmic nuclear profile area for histomorphometry and cytomorphometry.

	histological findings Grade 1		Grade 2		Grade 3		Inflammation	
	n	mean±SD	*n*	mean±SD	*n*	mean±SD	*n*	mean±SD
Histomorphometry								
LARGE	11	4.209±0.243	16	4.635±0.208	12	4.807±0.196	0	
DEEP	11	3.767±0.214	16	4.184±0.348	12	4.332±0.318	0	
SUPERF	11	3.492±0.238	16	3.840±0.248	12	4.048±0.375	0	
Cytomorphometry								
Pap smear	11	3.584±0.549	16	4.123±0.379	12	4.781±0.371	10	3.390±0.157
Feulgen smear	9	3.441±0.380	12	3.985±0.356	9	4.440±0.463	1	3.045
Pap section	11	3.424±0.260	15	3.758±0.220	11	4.020±0.229	10	3.428±0.142
Feulgen sect.	10	3.118±0.363	16	3.646±0.282	11	3.948±0.284	9	3.056±0.198

The values for the group of inflammatory cases are in the same range as for the grade-1 carcinomas, The significance of the differences between the four groups (see Table 4) was tested univariably, with the two-sided *t*-test. Between the group of inflammatory changes and grade-1 carcinomas, none of the variables proved to be significantly different. The results of the *t*-test for the bladder carcinomas are shown in Table 5. For all variables, there were significant differences between grade-1 and grade-2 tumors, and between low grade (grade 1 and 2) and high grade (grade 3).

Table 5. *t*-test results.

	inflammation-1	Grade 1-2	Grade 2-3	Grade 1+2-3
Histomorphometry				
LARGE		<	<	<
DEEP		<	=	<
SUPERF		<	=	<
Cytomorphometry				
Pap smear	=	<	<	<
Feulgen smear		<	<	<
Pap section	=	<	<	<
Feulgen section	=	<	<	<

$P<0.05$
= : not significantly different for mean nuclear profile area
< : value of first group significantly smaller value than second group

In Table 6 the correlation matrix for the variables of the bladder carcinomas is shown.

Grade	1.0000 (0)						
LARGE	0.7265 (39)	1.0000 (0)					
DEEP	0.5803 (39)	0.8461 (39)	1.0000 (0)				
SUPERF	0.6022 (39)	0.7398 (39)	0.7831 (39)	1.0000 (0)			
Pap smear	0.7430 (39)	0.6126 (39)	0.3874 (39)	0.5059 (39)	1.0000 (0)		
Feulgen smear	0.7156 (30)	0.6262 (30)	0.3429 (30)	0.5434 (30)	0.8942 (30)	1.0000 (0)	
Pap section	0.7123 (37)	0.6858 (37)	0.6191 (37)	0.6722 (37)	0.7582 (37)	0.7465 (28)	1.0000 (0)
Feulgen section	0.7211 (37)	0.6312 (37)	0.4726 (37)	0.5372 (37)	0.8721 (37)	0.8763 (28)	0.8023 (35)
	Grade	LARGE	DEEP	SUPERF	Pap smear	Feulgen smear	Pap section

All coefficients are positive and, with the exception of the coefficient between Deep and Feulgen smear, significantly differing from zero (α=5%, two-sided test). Within the histomorphometric variables, LARGE correlates best with grade, and of the cytomorphometric variables Pap smear is best. In Figure 4 these two are plotted for. all cases to obtain an impression whether classification is feasible.

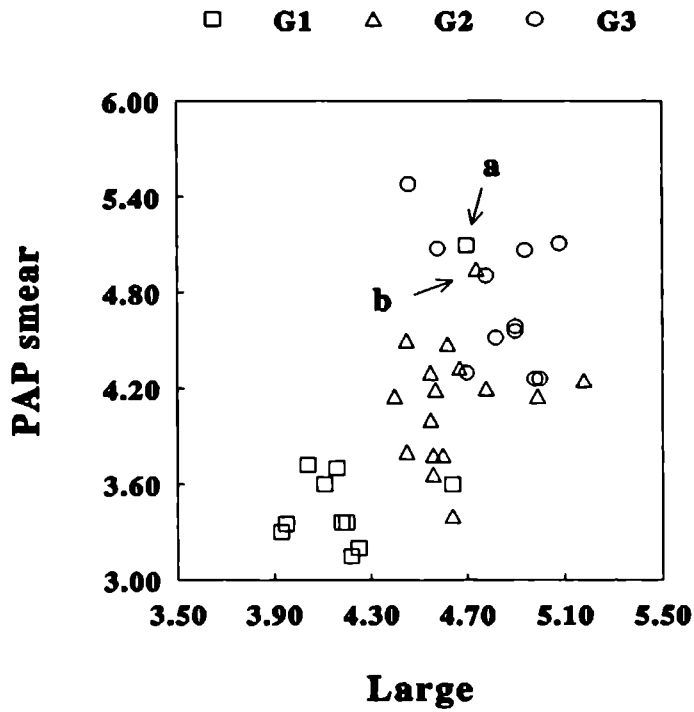


Figure 4. Scattergram of the log mean nuclear area values for LARGE with Pap smear and LARGE by histological tumor grade.

The results of the probit model for testing significance of additional information of cytomorphometry with regard to histomorphometry are given in Table 7.

Table 7. Significance of the additional information provided by cytomorphometry for the histomorphometry and vice versa.

Additional information in:	with regard to:	*n*	Significance
Pap smear	LARGE/DEEP/SUPERF	39	$P<0.001$
LARGE/DEEP/SUPERF	Pap smear		n.s.
Pap section	LARGE/DEEP/SUPERF	30	$P<0.05$
LARGE/DEEP/SUPERF	Pap section		n.s.
Feulgen smear	LARGE/DEEP/SUPERF	37	n.s.
LARGE/DEEP/SUPERF	Feulgen smear		n.s.
Feulgen section	LARGE/DEEP/SUPERF	37	$P<0.01$
LARGE/DEEP/SUPERF	Feulgen section		n.s.
All cytomorphometry	LARGE/DEEP/SUPERF	26	n.s.
LARGE/DEEP/SUPERF	All cytomorphometry		n.s.

Three of the four cytomorphometric methods add to the value of the histomorphometric data, but the converse is not true. Note that for the calculation of the significance of the additional information of all four cytological methods combined we had only 26 cases available. This may be the reason that the *P* value was over 0.05. In addition we found that combining cytological methods did not improve results. Using one out of the methods is sufficient and as the Pap smear is the easiest we preferred this method. The probability for high grade, using histomorphometry, can be estimated using the formula:

$$\Phi \ (-17 + 4 \times \text{LARGE} - 2 \times \text{DEEP} + 1 \times \text{SUPERF})$$

(see error curve of standard normal distribution). The probability for high grade using Pap smear can be estimated using the formula:

$$\Phi \ (-10 + 2 \times \text{Pap smear}).$$

RESULTS HISTOMORPHOMETRIC ANALYSIS

Table 8 shows the F values for the different features. No significant difference ($P > 0.05$) between grade 1 and 2 was calculated for the following features: 60 μm^2ER, 90 μm^2ER, FormPE, 5cER, and all texture features, except L1. For all morphometric and densitometric features there was a significant difference ($P < 0.05$) between low-grade (grade-1 and 2) and high-grade (grade-3) tumors.

Morphometry

Of the morphometrical features the 60 μm^2ER showed the highest F value (Table 8). The stereological calculated mtVOLUME had the second highest F value in the morphometry group. The form features FormELL and FormPE had lowest F values. The 60 μm^2ER and the 90 μm^2ER showed the highest mean values in grade-3 tumors. There was no significant difference for both features between grade 1 and 2 ($P > 0.20$). Both features, though, showed significant difference between low-grade (grade 1 and 2) and high-grade (grade 3) tumors ($P < 0.001$).

Densitometry

The 2cDI (Table 2) showed the highest F value of the densitometric features (Table 8). Figures 5-7 show the DNA histograms of examples of three different tumor grades. The difference between 5cER of grade 1 and 2 is not significant ($P > 0.05$). The values of 5cER differed significantly ($P < 0.001$) between low-grade (grade 1 and 2) and high-grade (grade 3) tumors (Figure 8). The grade-2 tumors could be divided into a group of 5 cases (55%) without nuclei with a DNA value exceeding 5c, and a group of cases (45%) with such nuclei. Except for significant differences in 2cDI and 5cER ($P = 0.025$), there were no significant differences between these two groups for the other features ($P > 0.15$).

Table 8. F-values for the features in the discriminant analysis. The features marked with an asterix are used in the canonical classification rule in the discriminant analysis.

Morphometry:	F-value	Wilks Lambda
1. rAREA	19.597	0.38945
2. mtVOLUME	20.095	0.38350
3. PERIM	14.447	0.46388
4. 60 μm^2ER*	24.368	0.33905
5. 90 μm^2ER	12.775	0.49456
6. FormELL*	8.757	0.58804
7. FormPE	4.277	0.74507
Densitometry:		
8. OD	3.609	0.77597
9. IOD*	43.103	0.22481
10. 2cDI*	56.005	0.27099
11. 5cER	33.627	0.18247
12. mtDNA	42.386	0.22774
Texture analysis:		
13. h1	4.110	0.75254
14. h2	3.212	0.79558
15. h3	1.898	0.86821
16. h4	1.329	0.90392
17. h5	1.216	0.91132
18. l1*	2.466	0.83521
19. l2	0.871	0.93483
20. l3	-	-
21. l4*	6.495	0.65808
22. l5*	0.926	0.93103

* features used in the classification function of the discriminant analysis

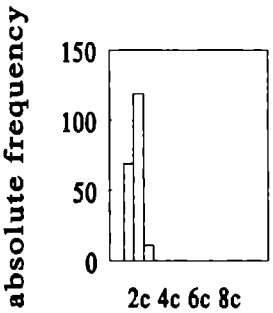


Figure 5. DNA-histogram of grade-1 tumor

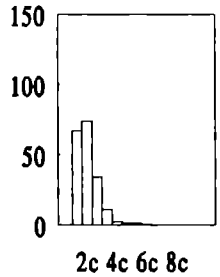


Figure 6. DNA-histogram of grade-2 tumor

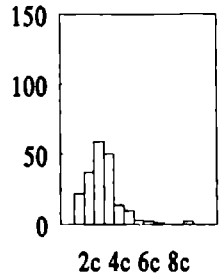
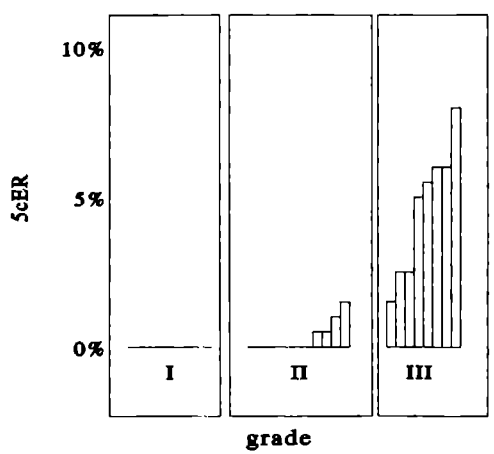


Figure 7. DNA-histogram of grade-3 tumor

Texture analysis

The texture features showed predominantly low *F* values (Table 8). The data indicated that higher tumor grades contain nuclei with more coarse and irregular chromatin patterns, but significance of these features in tumor grading is low.

Figure 8. Distribution of 5cER by tumor grade. Notice the two groups in the grade-2 tumors.



Multiparameter analysis

The discriminant analysis resulted in a class model containing seven features (out of the 22) listed in Table 9.

Table 9. Standardized canonical discriminant function coefficients. The order of the features is the step order in which the seven features are entered in the discriminant analysis.

Step	Function 1	Function 2
1. 2cDI	1.1568	-0.2443
2. L4	-0.8079	0.3957
3. IOD	0.0437	1.2197
4. FormELL	0.6024	0.0881
5. L1	0.5434	0.7314
6. 60 μm^2 ER	1.1568	-0.2443
7. L5	0.4929	-0.6048

Table 10. Classification results.

		predicted grade		
Actual grade	*n*	I	II	III
I	9	7	2	0
II	11	1	10	0
III	8	0	0	8

Of the seven features, two were morphometric, two were densitometric, and three were texture features. Note that the form feature FormELL was included. This model gave a classification rule of 89% correct classifications over three grades. All grade-3 tumors were correctly classified (Table 10). The final F value was 17.7194 (degrees of freedom 6 and 46). The scattergram of the two canonical discriminant functions is shown in Figure 9. We were aware that to use a stepwise discriminant analysis with 28 cases and 22 variables to select from was not ideal. However, as the final F value turned out to be highly significant ($P < 0.001$) the danger of overinformation[23] seems

to be limited in this case. To evaluate the value of the stereologically calculated features mtVOLUME and mtDNA, a discriminant analysis was performed using only these two features. The classification rule obtained this way predicted 79% of the cases correctly.

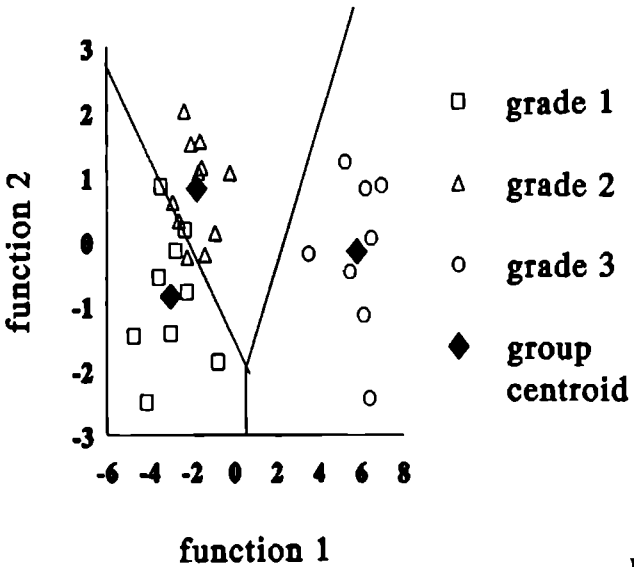


Figure 9. Scattergram of canonical discriminant function scores as presented in Table 9 (n=28).

DISCUSSION

The importance of grading of bladder carcinoma is well recognized, however, the question is how to achieve reproducible results. Colpaert et al. (1987)[24] showed that with precise criteria, with examination of the total section systematically field by field, and with recording of a grading per field, a better interobserver consistency can be obtained. The simple criterion of nuclear size (sometimes within a small subpopulation of cells) appeared to be a very important factor in the subjective

grading of histological sections. This is in accordance with our quantitative findings: of the histomorphometric features the value for LARGE nuclei correlated best with Grade. It seems that architectural features of the tumor, such as arrangement of nuclei, coalescence of papillae and whorl formation occurring increasingly in higher grades are closely correlated with nuclear size.[24] As for the histological grading of cancer of the larynx, there is also a close correlation between tumor architecture and nuclear size variation, both less well correlating with degree of keratinization.[25] It is likely that nuclear size as a discriminator is exclusively of value in tumors with a morphological continuum. Transitional cell carcinomas do represent a kind of tumor with increasing cell abnormalities in the higher grades.[4,10,11,26]

In this chapter we present our work on the grading of transitional cell carcinoma. First we use a single nuclear feature, nuclear size, and found it useful for tumor grading of bladder washings. An important point in the evaluation of a low number of cells (50) per slide is the selection of material (see Chapter 2). The selection criteria proposed in the cytomorphometrical grading of bladder washings use the cellular arrangements rather than the cellular atypia for selection of nuclei. Hence, the low number of measured nuclei is compensated by selection of the most representative tumor cells. In Chapter 2 the selection criteria will be tested on reproducibility and compared to at random analysis.

Nuclear size was of use in histomorphometric analysis as earlier shown by Ooms et al. (1983).[1] All three histomorphometric features used in the present study are closely correlated. LARGE (the best discriminator of grade) correlates best with the cytomorphometric features Pap smear (Table 6). The value for this feature depends on the presence of a subpopulation of cells with large nuclei in the histological sample. The fact that highly malignant cells with large nuclei are easily dislodge, thus are likely to appear in cytological material[27,28] probably accounts for this correlation.

For analysis of cytological material, bladder washings were preferred over voided urine. In particular in grade-1 and grade-2 carcinomas the harvest of tumor cells is much larger in washings. Moreover, material in bladder washings is more suitable for morphometry than voided urine.[8,9,30-32] The values for the four types of cytopreparatory techniques showed interesting patterns. The mean nuclear profile area values for the Papanicolaou stained cells (both in the smears and the cell blocks) were larger than those for the Feulgen-stained cells. It seems unlikely that staining itself changes the size of the nuclei.[29] We first postulated that the impression of the nuclear contour in the two staining procedures is different, influencing the manual measurements. Later

study, however,[33] revealed influence of Feulgen staining on nuclear size. It was postulated that the HCl step in the Feulgen-staining procedure resulted in nuclear shrinkage. The nuclear area in the smear preparations (both for the Papanicolaou and for the Feulgen staining) was generally larger when compared with those for the cell blocks. In the Papanicolaou staining, this was the case in 21 of the 27 low-grade carcinomas, whilst for all 12 high-grade carcinomas the values in the smears were higher. For the nine grade-1 carcinoma cases in which both smears and cell blocks were available the mean size difference was 8%, for the 13 grade-2 carcinomas 14%, and for the 9% grade-3 carcinomas 24%. Similar trends were observed for the Feulgen preparations. Physical factors such as nuclear rigidity are evidently important here and seem to vary between the different tumor grades.[29] Another reason for this difference in nuclear area can be found in the Holmes' effect. Holmes described a correlation between the mean nuclear area and the thickness of the section.[34] The fact that not all nuclei are cut in the equatorial plane results in a smaller mean nuclear profile area of the sections, compared to the smears. This was also found by Helander et al. (1985).[6] In the final evaluation, all four cytopreparatory techniques were equally good for morphometric grading. Since the Papanicolaou smear technique is the simplest of the four, it would be most suitable for morphometric grading of nuclear size. Considering the predictive value of nuclear DNA content and the vast development of image analysis equipment capable of analysis of multiple nuclear features, Feulgen stained cytological slides are a could alternative.

Statistical evaluation showed that histomorphometry does not add to cytomorphometry, but cytomorphometry does add to histomorphometry. No doubt, this can be explained by the selective exfoliation of discriminating cells with large nuclei. Since it is easier to obtain material for cytology, this is a highly rewarding result.

Patients A and B are interesting cases (see Figure 4). Both were examples of patients with recurrent low-grade superficial papillary carcinomas developing high grade carcinoma in situ during follow up.[35,36] The follow up results of these two patients are shown in Table 11.

Table 11. Case history of patients A and B (see Figure 4).

	Patient A	Patient B
1980	Histology Grade-1 tumor	-
1981	Histology Grade-1 tumor	Histology Grade-1 tumor
1982	-	Histology Grade-1 tumor
1983	Histology Grade-1 tumor	Histology Grade-1 tumor
1984	Histology Grade-1 tumor	-
1985	-	Histology Grade-2 tumor
1986	Cytomorphometry of bladder washing; Histology Grade-1 tumor	Cytomorphometry of bladder washing; Histology Grade-2 tumor
1987	Histology Grade-3 + CIS	Histology Grade-3 + CIS
1988	-	Histology Grade-3 invasive tumor

In both patients, the cytological bladder washing of 1986 reflected the true status of the urothelium, that is of a high grade carcinoma. In that year, easily visible concurrent low-grade papillary tumors were removed for histologic diagnosis, giving the false impression that the prognosis was relatively favorable. The grade-3 carcinomas in situ, (exfoliating the highly malignant cells in the bladder washings) were not diagnosed initially due to the fact that they were not macroscopically identified during cystoscopy. A year later, in 1987, the foci of high-grade carcinoma in situ were detected, probably partly due to the fact that at this moment there were no concurrent papillary carcinomas catching the attention of the cystoscopist. The cystectomy specimen (1988) of patient B contained extensive high-grade carcinoma in situ and many foci of invasive carcinoma. Patient A is receiving (1987) radiation therapy with little success. In none of the other low-grade papillary carcinoma cases was this reversal to high-grade cytology (due to carcinoma in situ) observed during follow up of 1-2 years.

Before focusing on the second study in this chapter we want to shed some light on the development of image analysis systems and their influence on the approaches of our studies. In the study discussed so far we applied an interactive morphometry system. Nuclei were selected based on criteria, the contour was manually drawn on the digitizer tablet, and calculation of a limited number of nuclear features was performed. Although inexpensive and easy to use, such a system clearly has its disadvantages. First, due to the manual selection and outlining of the objects only a

limited number of nuclei can be analyzed. Moreover, the system did not allow densitometric measurements or more complicated analyses of nuclear shape. Since these features play an important role in tumor grading[4,26,35] inclusion of these characteristics may enhance the value of the quantitative grading system. Therefore, for the second study in this chapter we applied the IBAS 2000 system.[12] This system enables analysis of multiple nuclear features including densitometric analysis.

Most quantitative studies using histological material, reported in the literature were performed on paraffin sections. We applied plastic sections for the following reasons: (1) a smaller section thickness[33,37], resulting in less overlapping nuclei[37]; (2) a more stable processing method, especially the absence of nuclear shrinkage in the hydrolysis step of the Feulgen staining.[3,33]

The disadvantage of using histologic sections is that slices of nuclei are measured, instead of entire nuclei. Nielsen and associates[38] estimated nuclear volume with point-counting and found only one of 35 patients with mean nuclear volume below 300 μm^3 dying of cancer within a 5-year follow up, whereas 18 of 19 patients with a mean nuclear volume above 500 μm^3 developed metastasis or died of cancer. The interactive nature of stereologic methods and the limited number of assessable nuclear features, however, overshadow advantages as simplicity and low costs of this technique. To recalculate the mean nuclear volume a stereologic approach was applied in the present study. Knowing the mean nuclear volume a calculation of mean nuclear DNA content could be made, considering the IOD in the formula. The two features (mtVOLUME, mtDNA) calculated this way classified 79% of the cases according to the tumor grade. In the multivariate approach, however, *F* values for the stereologic features (mtVOLUME, mtDNA) did not differ from the karyometric features (AREA, IOD) from which they were derived, moreover, the stereologic features did not provide additional information for the quantitative grading.

Besides this stereologic approach the three feature groups, morphometric, densitometric, and texture features were used in concert to predict tumor grade. Morphometry of the nuclear profile in sections and cytologic smears proved to be effective.[3,21,39] In the present study the morphometric features AREA, PERIMETER, 60μm^2ER, 90 μm^2ER, FormELL, and FormPE were used. In the first study of this chapter we found a correlation value of AREA with grade of 0.7211 in Feulgen-stained paraffin sections.[3] This is close to the value found in the present study: 0.728. Helander[37] showed that nuclear areas exceeding 90 μm^2 in GMA-embedded bladder-carcinoma material were almost exclusively found in grade-3 tumors: the percentages

of 39 and 9 for respectively 60 μm^2ER and 90 μm^2ER of grade-3 bladder tumors were remarkably well in agreement with the percentages of 40 and 10 found in the present study. These two features could very well discriminate between low-grade (grade 1 and 2) and high-grade tumors (grade 3).

Densitometry was used in the present study to assess the DNA content of the tumor cell nuclei, which proved an important feature for prognosis.[17,35,39-43] Nuclear DNA content was also valuable for the grading of bladder cancer by flow cytometry.[35,40,43-46] Flow cytometry is basically a cytologic method requiring separation of the cells and for above-mentioned reasons we preferred to perform cytophotometry on sections. The DNA histogram obtained by densitometry can be interpreted visually or using algorithms. We chose the algorithms 5cER and 2cDI according to Böcking.[17] The 2cDI has the highest *F* value of all features (Table 8), indicating that it is the most powerful discriminator of tumor grade. In 45% of the grade-2 tumors we found nuclei with more than 5c DNA content (suggesting aneuploidy). All grade-3 tumors had DNA-histograms that were grouped as aneuploid (based on 5cER). It should be noted that those aneuploid tumors with, for instance, a triploid subpopulation and lacking nuclei with DNA content over 5c, were not identified as aneuploid using 5c DNA as threshold. This theoretical problem seems to have no implications for bladder carcinoma, as can be deducted from the fact that Farsund and coworkers[45] found 43% of grade-2 tumors aneuploid by flow cytometry, compared to 45% in the current study. Tribukait and coworkers could also divide grade-2 tumors on base of proliferation and ploidy.[43] It is of importance to note that in the present study aneuploid grade-2 tumors were not identical to grade-3 tumors: their 5cERs and 2cDI values were significantly lower, so it seems ill advised to group these together.

Texture analysis can provide features describing chromatin patterns.[19,20,47-50] The Markovian features[19] were used in the present study and proved to be useful features of chromatin patterns in histologic and cytologic material.[20,48,50] In this context it is of interest that of the seven features selected in the discriminant analysis three were texture features. Hence texture features did contain useful information on tumor grade not provided by the other features.

Finally, with the classification rule in which seven morphometric, densitometric, and texture features are used in concert, 89% of the cases are classified correctly. All grade-3 tumors are classified according to the visual grading.

To obtain a clinical relevant grading system using the proposed quantitative analysis a continuous scale should be used. In that case each tumor can be assigned a

quantitative grading score. In this way the oversimplification of dividing bladder carcinomas into three grades can be avoided. Whether this really results in a useful clinical method remains to be tested on a large clinical material. The subdivision of the visually determined grade-2 tumors could validate the additional use of image analysis to pathologists grading.

We have to bear in mind, however, that the results of the presented studies are obtained in research laboratory settings where material processing and analysis was optimally controlled can not be extrapolated to clinical situations. Futher analysis of the grading techniques evaluated in this chapter with special regard to clinical applicability, therefore is mandatory.

In this chapter we presented the studies on the correlation between karyometric features and visual tumor grading in cytological and histological material of transitional cell carcinoma. Nuclear profile area measured in bladder wash cytology showed a good correlation with histological tumor grade and aided in two cases in the earlier diagnosis of high-grade carcinomas. Multivariate analysis of karyometric features in histological material revealed additional information of the karyometric features to visual grading. To analyze the predictive value of quantitative grading systems karyometric data should be correlated to clinical follow up rather than to visual tumor grade. Hence, in Chapter 3 and 4 clinically oriented studies will be discussed.

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Chapter 2

THE REPRODUCIBILITY OF KARYOMETRIC GRADING ROUTINES FOR BLADDER TUMORS

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ABSTRACT

The reproducibility of karyometric analysis of transitional cell carcinoma is studied. The inter-individual consistency is low ($r=0.55$ $p=0.0005$) when 50 nuclei were chosen at random. When we used selection criteria, based on cell grouping and cytological features of malignancy, the consistency between two technicians appears to be significantly higher ($r=0.90$ $p=0.015$). Not only inter-individual consistency increased using the selection, but the correlation with histological tumor grade significantly improved for both technicians also. Analysis of a higher number (100 or more) of randomly selected nuclei resulted in reproducibility comparable to selective analysis. The results show that cytomorphometry can be a method for grading of bladder carcinoma when an accurate selection of nuclei is applied or a larger amount of nuclei is analyzed. Hence, karyometric studies must contain a clear description of the way the nuclei for measurement are selection.

INTRODUCTION

Bladder neoplasms rank in the ten most common causes of cancer death.[1] Tumor grade correlates with prognosis,[2,3] and nuclear area significantly correlates with tumor grade.[4-6] These facts make morphometry a valuable method in the grading of bladder tumors.

Bladder washings of patients with bladder tumors often contain components of normal urothelium and non-malignant cells next to tumor cells.[4] Hence, the cytological grading of bladder cancer is based on those cells with the most pronounced anaplasia.[4] When measuring exfoliated cells in cytological material it is important to measure those cells that represent the true (histological) tumor grade. Due to practical limitations, like analysis time, only a low number of nuclei can be analyzed applying the interactive morphometric system as used in Chapter 1. This makes selection inevitable.[6] The problem with selection is the risk of subjectivity, decreasing reproducibility. For this reason selective morphometry can only be used if: 1. the criteria for the selection of nuclei are clear and 2. there is little inter-individual and intra-individual inconsistency.[9,10] In this chapter we test two different quantitative light microscopy settings for reproducibility. Using an interactive system (see Chapter 1), nuclear profile areas of 50 nuclei selected at random and according to microscopic criteria were measured. The reproducibility of automated image analysis of a larger amount of nuclei was tested for the system as described in Chapter 4.

Recent developments in image analysis techniques have resulted in a reduction in the time required for analysis of larger numbers of nuclei (100-500). Using these techniques, selection might be redundant since a larger random sample of cells in the cytological material will more likely contain tumor cells. Moreover, a larger random sample provides more accurate information on all cells in the material. In Chapter 4 we will discuss the application of automated karyometric analysis for the follow up of patients with superficial bladder cancer. Here, the reproducibility of automated image analysis of a larger amount of nuclei is tested.

We wanted to answer the following questions: 1. How reproducible are karyometric analyses of cytological material in interactive and automated image analysis systems; 2. Can selection of nuclei increase reproducibility; 3. Does selection influence predictive value of karyometric analysis.

MATERIAL AND METHODS

Material for interactive cytomorphometric study

The material consisted of 39 consecutive cases with the diagnosis of bladder tumor, graded histologically according to the WHO system. The histological material, obtained by transurethral resection was graded histologically and histomorphometrically according to the method described by Ooms et al.(1983).[10] The histomorphometrical grading served as the basis for the grading of the cytological material. Eleven were graded as grade 1, 16 as grade 2 and 12 as grade 3. The cytologic material was obtained by means of bladder washing with physiological saline solution.[11] The material was obtained in the Westeinde Hospital in The Hague. The earlier described fixation process was used.[8] The sediment obtained after centrifuging was smeared on slides and stained using the Papanicolaou method.

Material for automated karyometry study

Bladder wash material for the automated karyometric analysis was obtained by similarly rinsing the bladder with saline solution. Material was instantly fixed in 50% ethanol containing 2% polyethylene glycol (Carbowax), Cytospin (Shandon) centrifuged slides were obtained and stained according to Feulgen. Forty-eight at random selected samples in a population of 1460 samples were analyzed by three technicians.

Image analysis systems

Cytomorphometrical measurements were made using a microscope connected with a computer MOP-video (KONTRON). Fifty nuclei were delineated manually by the technician in each slide. Each slide was measured twice by the same technician. Both technicians did one measurement at random and one selecting the nuclei according to the described selection criteria. The cytomorphometrical measurements took approximately 15 minutes per slide. Automated karyometric analysis was performed on the image analysis equipment as described in Chapter 4. All slides were analyzed by 4 technicians. Analysis comprised recording of 50 randomly selected images per slide applying manual focusing of each image. After recording by the technician the images were sequentially analyzed without operator intervention. Each detected nucleus in the images was contoured and numbered. After the computer analysis each detected nucleus was visually screened and artifacts or faulty segmentated nuclei could

be deleted. Analysis of normal bladder wash samples indicated a somewhat lower Feulgen staining in normal lymphocytes compared to presumably diploid bladder mucosa cells. Application of the earlier described correction factor of 1.19 for lymphocytes was necessary using lymphocytes as references for normal 2c DNA content.[12]

Selection process in cytomorphometric analysis

The selection was as described in the first chapter for the cytomorphometric analysis and was conducted as follows:

Step 1.

First, the slide is screened for single, highly malignant cells. These cells always are found in the exfoliated material of high grade carcinomas.

Not all loose-lying cells are malignant though. Squamous cells and normal urothelial cells do occur singly separated from cell groups but can easily be recognized as benign.[13]

The cellular features of the single, high-grade malignant cells are as follows: a. cytoplasm: variably dense, with vacuoles and irregular cell shapes; b. nucleus: enlarged nucleus, with an increased N/C ratio (approximately >0.60, Boon et al. 1981),[14] eccentrically situated in the cytoplasm, irregular shape, prominent nuclear border, coarse chromatin pattern, multiple, irregular shaped, prominent nucleoli.

When single cells are present, with the above mentioned features, these are used for morphometry.

Step 2.

When no loose-lying cells with the described malignant features are present, the slide is screened for small papillary cell groups.

Two types of small papillary cell groups are selected:

1. loose clusters of only 5 to 15 clearly malignant cells

2. small groups of less than 50 cells with round nuclei with little nuclear overlapping.

Step 3.

When these two types of cell grouping are not found, the slide is screened for large papillary groups. These consist of more than 50 cells, with smooth outlines, consist of many nuclei and are often very dense making measuring only possible at the edges of the cell groups.

Karyometric features

In the cytomorphometric analysis using the MOP-videoplan (Chapter 1) only nuclear profile area was measured. The reproducibility of the automated karyometric analysis was tested for the karyometric features selected in the logistic regression function as calculated in Chapter 4 (2c Deviation Index and BEN, a nuclear shape feature, see Table 2 Chapter 1) and nuclear profile area and the number of analyzed nuclei per sample.

Statistical analysis

As in our earlier studies[8,14] we found a strong linear dependency between standard deviation and mean nuclear area (mean of correlation in the four groups: 0.83). The statistical analysis of the data is complicated by the fact that a systematic correlation exists between standard deviation and mean value. In an earlier histomorphometric study Ooms et al. (1983b)[10] also found this correlation. We attempted to eliminate this correlation by applying a variance-stabilising transformation on the original data and subsequently calculated the standard deviation. In this way the standard deviations do not contain information about the mean values and for this reason can be neglected. We tested this method and found it acceptable. As variance-stabilizing transformation we used the logarithm of the mean value.

In order to investigate the inter-individual reproducibility of a certain factor when measured by two different technicians using the same protocol, one should at least in our opinion, pay attention to the following guidelines: 1. The measurements should be highly correlated. How large correlation coefficients should be is a somewhat subjective affair. We proposed that $r>0.90$ or $r>0.95$. An interpretation of r is that the bivariate scatterplot is shaped as an ellipse for which the length of minor axis l_1 is $(1-r)^{1/2}/(1+r)^{1/2}$ times the length of the major axis l_2 (for $r=0.90$ $l_1:l_2=1:19^{1/2}$, for $r=0.95$ is $l_1:l_2=1:39^{1/2}$). We used the Fisher's z-transformation to obtain a test statistic. 2. The measurements should be exchangeable. This implies, among other things, that the measurements performed by one technician might have been performed by any other technician just as well. This is tested by assuming joint normality of (X,Y), where X is the random variable describing the measurement of technician A and Y that of technician B and using the GLR statistic for testing whether (X) = (Y).

In order to test whether the inter-individual sample correlation coefficient increases significantly due to the selection process we applied a divided sample approach (to obtain independent samples) and use Fisher's z-transformation to obtain

a test statistic.

To test the increase in consensus between cytomorphometry and histomorphometry when using selection let X denote a certain factor measured without selection and let Y denote the same factor with selection of nuclei to measure. In order to test whether X and Y are equally correlated with G (=grade) we postulate (to avoid statistical complexity) the ratio of variance of X to that of Y to be known and equalling, τ^2, say. Now testing H: (G,Y) = (G,X) is equivalent with testing H': $(G,Z_\tau)=0$, where $Z_\tau=X-_\tau Y$. Under rather general conditions we have that under H' [15]

$$T = (n-2)^{1/2} \frac{r_{G,Z}}{(1-r^2_{G,Z\tau})^{1/2}} \quad t_{n-2}$$

Now we replace the nuisance parameter τ^2 by its obvious sample estimate to obtain an approximate test.

For statistical analysis of reproducibility of the automated measurements the SPSS/PC+ 4.0 software was applied. A selection was made among the karyometric features. Besides nuclear profile area the karyometric features selected for the karyometric score for bladder washings, as described in Chapter 4, and the number of detected nuclei in 50 images were investigated for reproducibility.

RESULTS

Reproducibility of cytomorphometric analysis. For reasons indicated above we used the mean log nuclear area as a potential factor. Note that we have essentially four data-sets, namely the measurements of two technicians (A,B) each of which measured once with and once without selecting the nuclei (see Table 1).

Table 1. The log mean variables and standard deviation of nuclear area for the two technicians

	Grade 1.			Grade 2.			Grade 3.		
	n	mean	SD	n	mean	SD	n	mean	SD
technician A									
selective	11	3.58	0.14	16	4.17	0.16	11	4.67	0.25
at random	11	3.66	0.19	15	3.95	0.20	11	4.12	0.17
technician B									
selective	5	3.34	0.12	16	4.20	0.29	11	4.74	0.27
at random	5	3.61	0.17	16	3.86	0.20	11	4.21	0.21

The measurements based on the selection process gave rise to a significantly larger sample correlation ($P<0.001$, based on the divided sample test statistic) than the at random measurements. However, the hypothesis of exchangeability is still somewhat contradicted, although to a lesser extent than the exchangeability of the at random measurements (see Table 2).

Table 2. Results regarding the reproducibility of selective and non-selective morphometry. (two technicians).

selective	r	0.90
	P-value	0.015
non-selective	r	0.55
	P-value	0.5*E-3

Not only does the selection improve reproducibility, it also increases the consensus of the technician (cytomorphometry) and pathologist (histomorphometry), in the sense that the sample correlation coefficient between the grade and the mean log area increases significantly (Table 3).

Table 3. Correlation between histological grade and the log mean nuclear area.

	selective	non-selective	significance of increase
technician A	(n=38)	(n=38)	
	0.93	0.66	*
technician B	(n=33)	(n=34)	
	0.86	0.50	**

(*= 0.05>P>0.01 and **= 0.001>p>0.001)

To illustrate the above discussed reproducibility and consensus of the pathologist and technician the outcomes of the at random measurements of both technicians are displayed in Figure 1a, whereas those based on the selection process are displayed in Figure 1b.

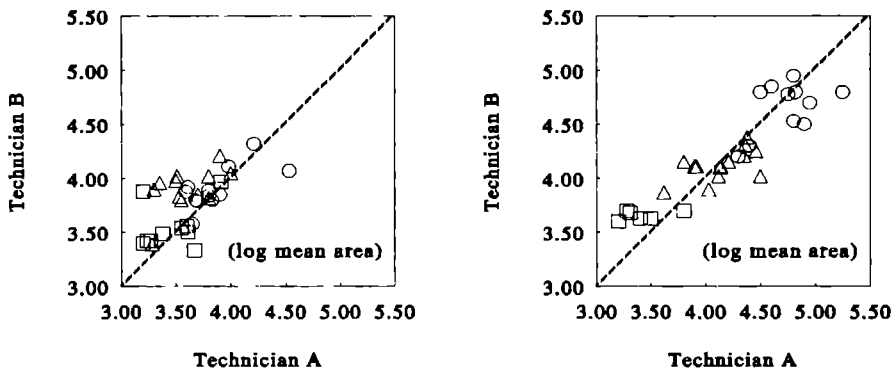

Figure 1. Correlation of nuclear profile area (log mean value per sample) between two technicians for random (a) and selective analysis (b). The different histological tumor grades are marked (grade 1: square, grade 2: triangle, and grade 3: circle).

Reproducibility of karyometric analysis. The correlation of analysis of nuclear profile area, number of analyzed nuclei per sample, nuclear shape (BEN), and the 2c Deviation Index among three technicians is presented in Figure 2 and 3 (Technician A vs B: n = 37; Technician A vs C: n = 28; Technician B vs C: n = 45).. In Table 4 the

correlations values between the measurements of the different technicians are shown. All correlations were significant and larger than 0.75, except for the 2c Deviation Index. This was caused by the large number of samples with 2cDI values smaller than 1. In this group low correlation values between the three technicians was found. In routine application this is of minor importance since distinction of low 2cDI values (< 1.0) is of no clinical importance (see Chapter 4). The correlation values for nuclear profile area are comparable to those found for selective cytomorphometry. The random selection of images could cause differences in the analyzed number of nuclei per sample between technicians. Correlation of the number of nuclei analyzed, however, was high for all three technicians (Table 4). In samples where only a low amount of nuclei (<100) could be analyzed, the differences in nuclear profile area between the technicians were significantly higher ($P<0.05$) compared to analysis of larger numbers of nuclei per sample.

Table 4. Correlation of karyometric analyses performed by three technicians. Two karyometric features are chosen regarding the prognostic evaluation as described in Chapter 4 (BEN and 2cDI). Nuclear profile area (NPA) is shown for comparison with the cytomorphometric analysis. Since measurements were performed at random in 50 images the number of nuclei detected in these images (NUCLEI) was also studied for the three technicians.

	NPA		NUCLEI		BEN		2cDI	
	R	*P*	R	*P*	R	*P*	R	*P*
Technician								
A vs B (n=37)	0.892	0.01	0.861	0.01	0.801	0.01	0.692	0.01
A vs C (n=28)	0.821	0.01	0.925	0.01	0.821	0.01	0.699	0.01
B vs C (n=45)	0.811	0.01	0.843	0.01	0.837	0.01	0.724	0.01

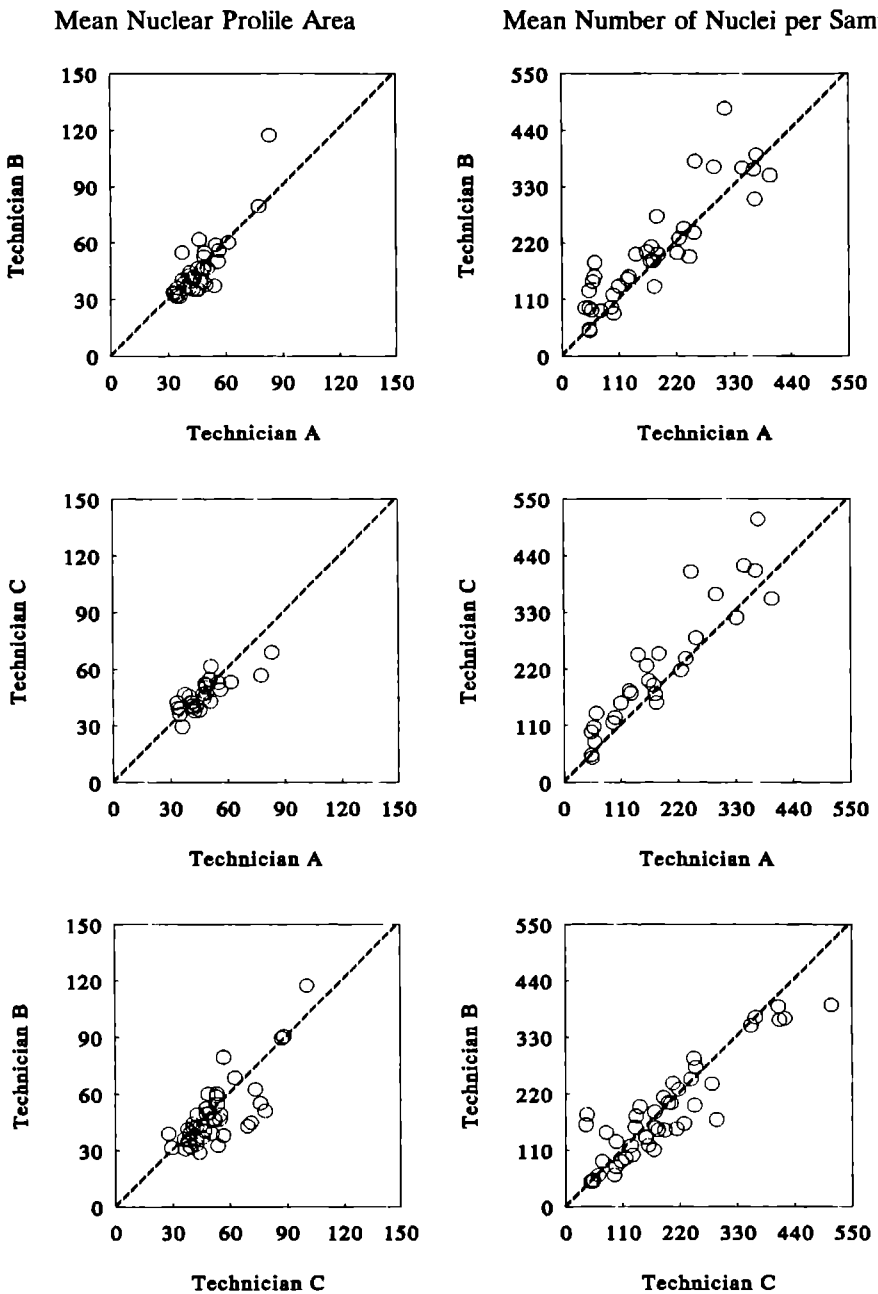

Figure 2. Mean nuclear profile area and mean number of nuclei analyzed per sample compared between the three technicians.

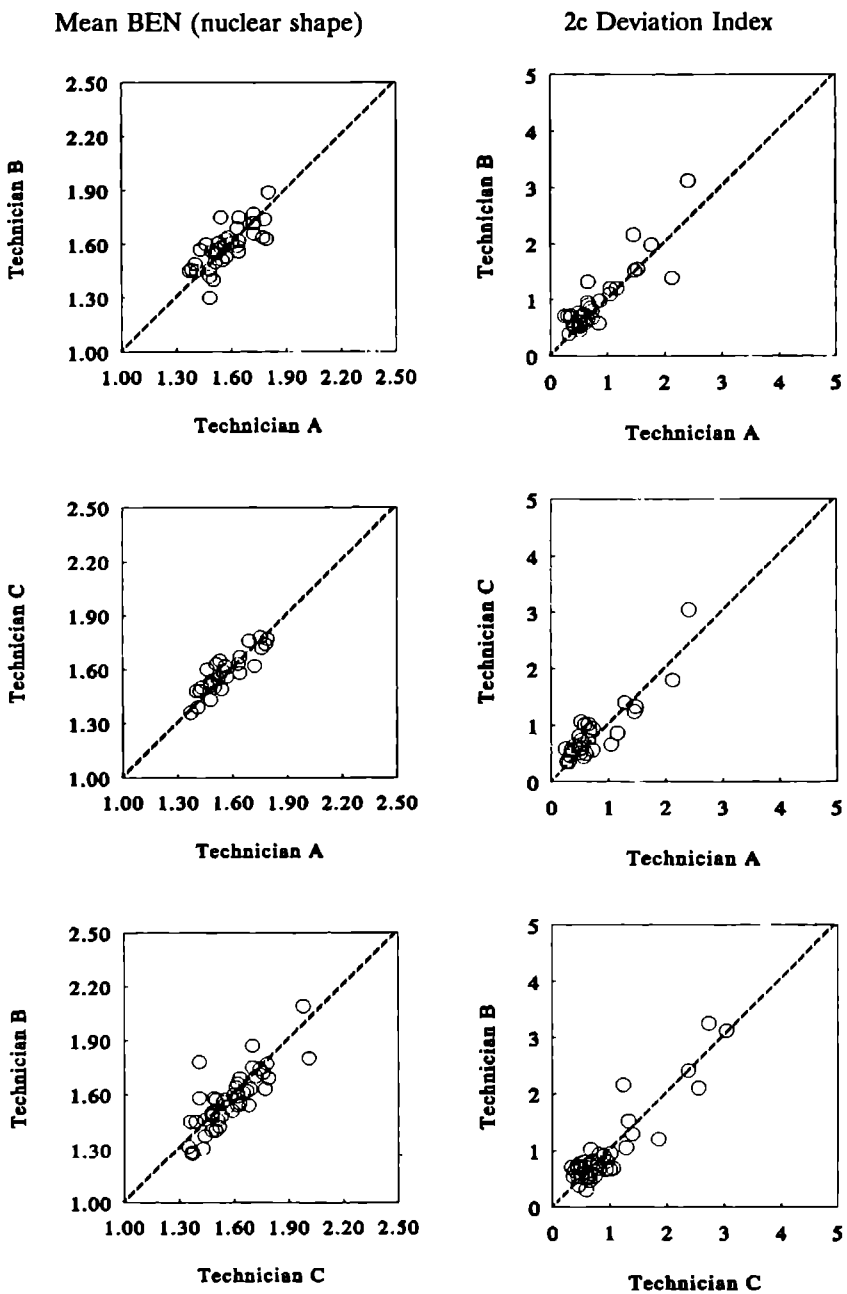

Figure 3. Mean BEN value and mean 2cDI per sample compared between the three technicians.

DISCUSSION

Urinary cytology is a valuable tool for the follow up of patients with superficial bladder cancer.[16,17]

Cytomorphometry of cells in the urine is used as a method to achieve a more reproducible way of cytological grading.[8,9] In an earlier study we found a correlation value of 0.743 for cytomorphometrically determined nuclear area with histological tumor grade.[8] A possible explanation for an increase in nuclear area with tumor grade is the increase in amount of DNA in the tumor nucleus. Helander et al. (1985)[18] found that grade-1 tumors were always diploid and grade-3 tumors consisted of aneuploid nuclei. The amount of DNA measured by flow cytometry shows a positive correlation with nuclear size.[18,19]

Colpaert et al. (1987)[4] showed that subjective grading is influenced by a small number of larger nuclei, representing tumor grade. They found that 50 percent of the nuclei in grade-3 tumors were within normal values.[4] If nuclei, chosen at random are measured a high percentage of 'non-representative' cells will be analyzed. Hence, in our data we found a lower correlation of karyometric analysis of nuclear profile area and histological tumor grade when samples were analyzed randomly compared to selection of nuclei based on cytological selection criteria.

The selection criteria applied were derived from interpretation criteria in visual cytology.[4] When screening the slide at low magnification three different types of cell grouping can be recognized: 1. single malignant cells; 2. small papillary groups and 3. large papillary cell groups. This division forms the basis of the selection process. When single malignant cells were present, this indicated loss of inter-cellular cohesiveness a feature of malignancy and present in grade-3 carcinomas.[20-22] Small papillary cell groups were present in grade-2 as well as grade-3 tumor material. Exclusively large papillary cell groups occurred mainly in grade-1 tumors. This kind of cell group frequently was found in tumors of higher grade but then always next to small cell groups or single cells. The number of papillary groups in the material depends on both the size and the location of the tumor.[22] Low grade tumor cells, characterised mainly by cell groups and less malignant cellular features are selected in step 3.

A repeatable and reliable selection of nuclei can be achieved by carefully analyzing the cellular features accompanying malignancy. Many studies have discussed

cellular features used in the grading of bladder tumors.[4,5,23-25] Ooms et al. (1983b)[10] described a histomorphometric grading system in which selection is directed by the location of the cells in the tumor. They discriminated: DEEP; SUPERFICIAL; and LARGE nuclei in bladder tumors and found a significant correlation between histological tumor grade and nuclear profile area.[10] In a following article they stress the importance of a clear selection of nuclei and show that the histomorphometric grading has better inter-individual consistency than the histological grading.[9] Blomjous et al. (1988)[26] selected visually only the 10 largest nuclei in histological sections of bladder cancer and found higher recurrence and progression rates in patients with a mean nuclear profile area over 95 μm^2 compared to patients with smaller nuclei. For the selection of cells for quantitative analysis in urinary sediment Koss et al. (1980)[27] developed a selective mapping algorithm[28] for automated detection of cells suitable for diagnosis at low resolution. Selected cells were then entered in high-resolution image analysis routines. The difference between the above mentioned (cytological) criteria[22,24,25] and the method used in the present study is the fact that in the current study, attention is paid to the cellular arrangements first. As the cell grouping is a feature that can be appreciated at low magnification this was used as the first step in the present analysis. The reason for this attention to the cell groups is that it simplifies screening and makes a graded analysis of the material possible. Individual cellular features become important in the second phase of selection.

The described cytological criteria in combination improved both correlation with histological grade and interindividual reproducibility. For this reason we conclude that when analyzing a low number of nuclei in cytological material manually selection criteria are mandatory. Two issues, however, remain:

- Selection of cells according to criteria requires subjective interaction, increasing the number of analyzed cells might make selection redundant.

- Cytomorphometric analysis correlated with histological tumor grade, the latter, however, is not necessary correlated with tumor behavior, e.g. recurrence and progression. Since, bladder tumors often occur on multiple sites of the bladder, it can be regarded as general disease of the urothelium resulting in localized lesions. Although we have to mention that controversy on this point exists,[29,30] we assumed that cytological characteristics of 'normal' urothelium in patients with bladder cancer might reveal pre-malignant changes that predispose for later tumor recurrences.

To study these phenomena we conducted the study as presented in Chapter 4 of this thesis. Here we present the results on the analysis of the reproducibility of the

applied karyometric analysis. Several differences between our first morphometric studies of nuclear size and the automated karyometric analysis bladder washings exist. First, due to technical limitations the interactive image analysis equipment (KONTRON) in the early studies was capable of analyzing only a limited number of nuclei, whereas the system used in the study in Chapter 4 enabled analysis of several hundreds of nuclei in a similar amount of time. Second, the number of features per nucleus was higher in the later study. Third, besides analysis of correlation with histological grade, correlation with follow up data was pursued in the later study.

Since we want to develop a clinical applicable quantitative analysis of cytological material we aimed at reducing time for analysis and subjective influences by analysis of randomly selected nuclei which could be conducted in an automated setting. No selection criteria were applied and only artifacts or out-of-focus nuclei were deleted. Moreover, this technique provides karyometric information of 'normal' bladder mucosa. The number of nuclei analyzed per sample influenced consistency of the measurements. For reproducible measurements we conclude from our data that at least 100 nuclei per sample should be analyzed when at random selection is performed. Reproducibility of the analysis conducted this way showed to be comparable to those obtained by selective cytomorphometry of a low number of nuclei. In Chapter 4 we will discuss the correlation with clinical findings. Anticipating on these results we mention here the fact that based on karyometric analysis a prediction of tumor recurrence in tumor-free samples is possible. This suggests that the criteria, as successfully applied for the detection and grading of tumors in cytological material may not be sufficient for the prediction of later tumor development since these changes occur in 'normal' urothelial cells.

This study shows that: 1. Analysis of a low number of nuclei in bladder washing material is useful for grading of bladder carcinoma only when careful selection of nuclei is applied. 2. The selection process, based on cellular arrangement in the cytological material increases reproducibility and gives a better correlation with histological tumor grade. 3. Analysis of a larger amount of cells selected randomly reached acceptable reproducibility and can be an alternative to subjective selection criteria.

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Chapter 3

KARYOMETRY IN RECURRENT SUPERFICIAL UROTHELIAL CELL TUMORS OF THE BLADDER

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ABSTRACT

Transitional cell carcinoma of the bladder shows a high recurrence rate after local treatment. Progression to higher stage occurs in 10-30% of the recurrent tumors and early detection of potentially progressive tumors is important. In the current study morphometric, densitometric, and chromatin texture features of nuclei of superficial bladder tumors (pTa-T1) were studied to determine the value of karyometric features in the prediction of tumor progression. Seventy-two histological samples of 36 patients, consisting of both the primary and the first recurrent superficial tumor were analyzed. Patients were divided into two groups: those with tumor-progression, defined as an increase in tumor stage or occurrence of metastatic disease, and those without. Discriminant analysis on 4 karyometric features resulted in correct prediction of prognosis of 78% and 97% in the primary and recurrent tumors respectively. Tumor grade and stage did not offer additional information concerning prognosis. Karyometric analysis of recurrent superficial transitional cell tumors can be useful in selecting patients that need more aggressive therapy. However, karyometric tumor characteristics of recurrent tumors were more predictive of progression than those of the primary tumor, whereas tumor grade was not different. Hence, continuous evaluation of the karyometric features is necessary for early detection of an increase in the malignant potential of the tumor.

INTRODUCTION

Superficial transitional cell carcinoma (TCC) of the bladder account for approximately 80% of all newly diagnosed tumors of this organ.[1] The tumor is treated by transurethral resection with or without intravesical instillation with chemo and/or immunotherapeutic agents. However, 30%-90% of the tumors recur depending on grade, multifocality,[1] and the treatment modality. Thiotepa resulted in a 49% recurrence rate.[2] Intravesical Epodyl therapy resulted in recurrence rates of 21 to 50%,[3] adriamycin showed recurrences in 40-70%,[4,5] and mitomycin C in 10-20% of the cases.[6] BCG instillations after TUR reduced the recurrence rate to 22% in a study by Shinka and associates (1990),[7] but seemed of no value for reducing the number of recurrences in stage T_1 tumors. The multifocal occurrence of bladder tumors suggests a general disease of the urothelium rather than a localized process. This can be an explanation for the high recurrence rate. Although superficial at the time of diagnosis, 10-30% of these tumors become invasive or metastasize in the course of the disease.[8] In case of frequent recurrences and multiple tumors, progression rate can be as high as 83%.[9]

Early identification of patients with progressive superficial bladder tumors has implications for the treatment, since a more aggressive treatment is indicated for these patients. Nuclear features of tumor cells in transitional cell cancer showed predictive value for recurrence and prognosis.[10,11] Nuclear profile area, its standard deviation, and DNA content (2cDI and 5cER)[12] correlated best with visual assessed tumor grade.[10,11,13-17] Ooms and associates (1983)[15] introduced a quantitative grading system with measurements of superficial, large, and deep cell nuclei separately in histological samples. De Prez and associates (1990)[13] described the use of an image analysis system (Samba 200) measuring morphometric, densitometric, and chromatin pattern features. These quantitative findings correlated well with visual grade. In the current study we analyzed whether nuclear features quantified by image analysis can play a role in predicting the prognosis in recurrent superficial bladder tumors. To test image analysis as a tool for patient follow up, the technique was applied in consecutive samples in order to compare primary and recurrent tumor of the same patient.

MATERIAL AND METHODS

Patients

Thirty six patients with superficial bladder tumors (stage Ta-T1) underwent complete transurethral resection (TUR) of the tumor and were treated with fifteen intravesical instillations of adriamycine. Material of primary and first recurrent tumor was available. Mean follow up was 4.8 years (3-10 yrs). The patients were divided into two groups: patients with progressive and non-progressive tumors during follow up. Tumor progression was defined as an increase in tumor stage to T2 or more or the appearance of metastases. Considering the relatively small number of patients, time to progression was not taken into the analysis.

Material

Paraffin-embedded, formalin-fixed TUR material was available of all patients from the primary and recurrent tumor. Four μm sections were cut, deparaffinized in xylene, rehydrated, and stained according Feulgen-Schiff (hydrolysis in 5 N HCl for 60 minutes and 30 minutes in Schiff reagent (Merck, Darmstadt, Germany) at room temperature). Haematoxylin stained slides were used by the pathologist to mark the tumor areas of interest. The marks were copied to the Feulgen-stained slides.

Quantitative microscopy

Image analysis was performed with a VS100-AT framegrabber board (Imaging Technology, Woburn, USA) in a personal computer (Compaq 386s). A videocamera (HCS-CCD, MXR, Vision Technology, Eindhoven, The Netherlands) connected to an Axioskop light microscope (Zeiss, Oberkochen, Germany) was used to record the images. Ten randomly selected images per slide were measured in these areas with a 40 times objective (pixel size 0.024 μm2). Analysis of one image took 3 minutes. The nuclear features measured are described in Table 2 and 3 of Chapter 1. The 5c exceeding rate (5cER) and 2c deviation index (2cDI) were calculated, with 30-50 lymphocytes as internal reference. The 5cER represents the percentage of definitely aneuploid cells, the 2cDI the (mean square) deviation from the diploid value.[12] Software used was written in TIM (TEA, Dordrecht) an image analysis language offering several basic modules for image recording, handling, and analysis. Additional software was written in TURBO-Pascal for chromatin texture analysis, Freeman chain code analysis for shape description, and data handling.

Prior to image segmentation, the image was corrected for shading and a median filter was applied. Selection of nuclei was primary based on size and values for maximal bending energy (BEN), in order to eliminate overlapping nuclei and

artifacts.[18] Visual inspection of the images overlaid by contours and numbers of the selected nuclei enabled screening for out-of-focus cells or artifacts.

Of each feature the mean, standard deviation (SD) and 90th percentile was calculated. The measurements were divided into three groups: 1. values of primary tumor; 2. values of recurrent tumor; and 3. differences in values between primary and recurrent tumor.

The SPSS/PC+ package (SPSS, Chicago, US) was used for statistical analysis. Mann-Whitney U-test and discriminant analysis were applied. To reduce the number of features in the discriminant analysis a selection of features was made based on results from the Mann-Whitney U-test, results from earlier studies,[18] and correlation with tumor grade. The features selected this way were: the 90th percentile of the nuclear profile area, the SD of MAC (nuclear shape feature; the standard deviation represents the degree of nuclear polymorphism), 2cDI, and the mean of Markovian feature H3, a chromatin pattern feature, based on the co-occurrence matrix. These features describe the presence of very large nuclei (NIN of NPA), the presence of nuclear polymorphism (SD of MAC, standard deviation of MAC, a nuclear shape feature), the variance in DNA content (2cDI), and uneven distributions in chromatin pattern.

RESULTS

Progression and grade and stage

Twenty-three patients did not show tumor progression after the recurrent superficial tumor. Progression was found in 13 patients during follow up (33%). Histological tumor grade in the primary-tumor group was not significantly different from the recurrent-tumor group ($P>0.05$). Whereas tumor grade of the recurrent tumor was significantly higher in progressive tumors ($P<0.01$, chi^2-test), tumor grade was not correlated with progression in the primary tumor. Twenty-two percent of the tumors showed an increase in tumor grade in the recurrent tumors, which is in agreement with other studies.[19] All tumors with a decrease in tumor grade between the primary and the recurrent lesion did not progress, in contrast to 4 of 8 cases which showed an increase in tumor grade ($P=0.03$, chi^2-test) and progressed. In agreement with Kaubisch and associates[20] we did not find progressive tumors in patients with only grade-1 lesions as recurrent tumor, whereas only one patient with a grade-1 primary tumor showed progression.

Stage of the primary and recurrent tumor (T_a or T_1) was not significantly different. Of the recurrent cases tumor stage was higher (T_1) in the tumors that subsequently progressed ($P<0.05$, chi²-test). Tumor stage of the primary tumor did not differ between progressive and non-progressive tumors. There was a tendency to higher tumor grade in stage-T_1 tumors as compared to stage-T_a, although, this was significant neither in the primary, nor in the recurrent tumor.

Karyometry and grade and stage

Several karyometric features showed a correlation with histological tumor grade in the primary as well as in the recurrent tumors. Of the morphometric features, the mean nuclear profile area increased with tumor grade as did its coefficient of variation, indicating a higher anisokaryosis in the higher tumor grades. Grade-3 tumors had significantly higher values for 2cDI and 5cER, however, several cases of the high-grade tumors had values within normal range. Features in both primary and recurrent tumors showed similar correlations with tumor grade. The results of the discriminant analysis resulted in 83% correct classifications. Karyometric features could not discriminate between stage-T_a and stage-T_1 tumors.

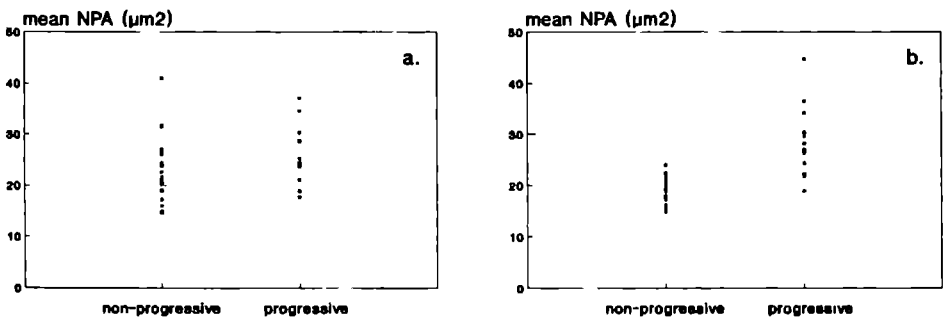

Figure 1. Nuclear profile area in primary (a) and recurrent (b) tumors of patients with progressive and non-progressive follow up.

Karyometry and progression

The results of the Mann-Whitney U-test showed a significant difference ($P<0.005$) between progressive and non-progressive tumors for 4 of the tested karyometric features in the primary tumors and 19 features in the recurrent tumors (Table 1 and

Figure 1).

Table 1. Karyometric features that were significantly different ($P<0.001$) between progressive (P) and non-progressive (NP) tumors in primary and recurrent tumors expressed as P-values from Mann-Whitney U-test. When both primary and recurrent tumor features were not significant, the feature is not shown.

	sign. of difference between P and NP in prim. tumor	sign. of difference between P and NP in recurr. tumor	sign. of difference between P and NP in difference values*
- mean NPA	n.s.	$P<0.0001$	$P<0.001$
- SD NPA	$P<0.001$	$P<0.0001$	n.s.
- NIN NPA	n.s.	$P<0.0001$	n.s.
- CV NPA	$P<0.001$	$P<0.0001$	n.s.
- mean PERI	n.s.	$P<0.0001$	n.s.
- SD PERI	$P<0.001$	$P<0.0001$	n.s.
- NIN PERI	n.s.	$P<0.0001$	n.s.
- mean MAXD	n.s.	$P<0.0001$	n.s.
- SD MAXD	n.s.	$P<0.0001$	n.s.
- NIN MAXD	n.s.	$P<0.0001$	n.s.
- mean FPE	n.s.	$P<0.001$	$P<0.001$
- mean MBEN	n.s.	$P<0.001$	n.s.
- SD MBEN	n.s.	$P<0.0001$	n.s.
- NIN MBEN	n.s.	$P<0.001$	n.s.
- SD MAC	$P<0.001$	n.s.	n.s.
- NIN MAC	n.s.	$P<0.001$	n.s.
- CV MAC	$P<0.001$	$P<0.0001$	n.s.
- mtDNA	$P<0.001$	$P<0.001$	n.s.
- mtVOLUME	n.s.	$P<0.001$	n.s.
- mean IOD	n.s.	$P<0.001$	n.s.
- SD IOD	n.s.	$P<0.001$	n.s.
- NIN IOD	n.s.	$P<0.0001$	n.s.

*) difference values are the differences of the karyometric feature values between primary and recurrent tumor. Or, in other words, the changes in the karyometric features of the recurrent tumor compared to the primary lesion.

- SD = standard deviation
- NIN = 90^{th} percentile
- CV = coefficient of variation (ratio of SD and mean)

Multivariate analysis

The Wilks method was used in a leave-one-out stepwise discriminant analysis (F to enter 3) to calculate a canonical linear discriminant function using the four selected feature values to discriminate between progressive and non-progressive tumors. The correct classification based on the selected nuclear features was highest in the recurrent tumors (97%). All non-progressive (NP) tumors (n=23) and 12 of 13 (92%) progressive (P) tumors were classified correctly (Table 3 and Figure 2b). Based on the primary tumors, a correct prediction of prognosis was obtained in 18 of 23 cases (78%) with no tumor progression (Table 2 and Figure 2a).

Table 2. Classification result discriminant analysis karyometric features for prediction of tumor prognosis of samples of the primary tumors. (method: Wilk's; F-to-enter = 3).

Standardized canonical discriminant function ($P<0.001$)
Dscr. Score = (1.20846 * SDMAC) + (1.00225 * H3)

	Predicted Prognosis	
	NP	P
Follow-up		
NP (n=23)	18 (78.3%)	5 (21.7%)
P (n=13)	3 (23.1%)	10 (76.9%)
correctly predicted:	77.78% (28/36)	

Table 3. Classification result discriminant analysis karyometric features for prediction of tumor prognosis of samples of the recurrent tumor. (method: Wilk's; F-to-enter = 3).

Standardized canonical discriminant function ($P<0.0001$)
Dscr. Score = (0.88226 * SDMAC) + (1.11261 * 90^{th} NPA)

	Predicted Prognosis	
	NP	P
Follow-up		
NP (n=23)	3 (100%)	0 (0%)
P (n=13)	1 (7.7%)	12 (92.3%)
correctly predicted:	97.4% (35/36)	

NP = non-progressive tumors
P = progressive tumors

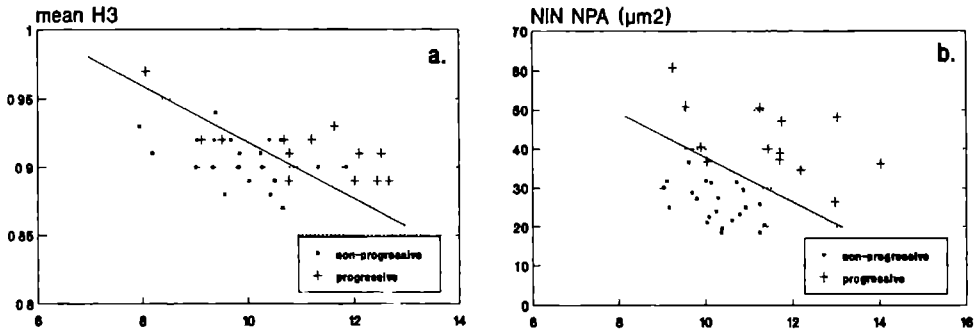

Figure 2. Features selected in discriminant analysis on base of F-value for the primary and recurrent tumors. The line represents the zero-scores of the canonical discriminant function (primary (a) and recurrent tumor (b)). Notice the different karyometric features selected in the discriminant analysis for the primary and recurrent tumor group: SD of MAC was a useful feature in both groups; best classification was obtained of SD MAC in combination with NPA (nuclear profile area) in the recurrent tumors (b) and in combination with mean H3 (nuclear chromatin pattern) in the primary tumors (a).

Ten of 13 tumors (77%) with tumor progression were classified correctly (Table 3). This resulted in a correct classification of 78% and 97% for the primary and recurrent tumors respectively (Figures 2a and 2b). When the discriminant function found in the recurrent tumors was applied on the primary tumor group the percentage of correctly classified cases did not change (Figure 3).

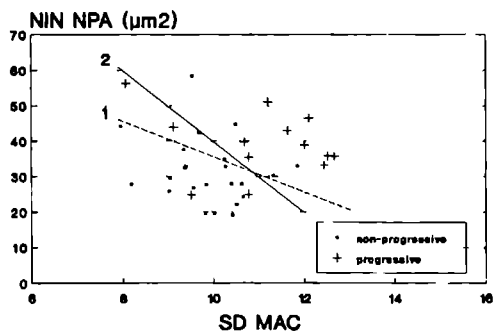

Figure 3. Canonical discriminant function (df) of the recurrent tumor (1) plotted in the primary tumor group. Line (2) represents the zero df scores when the analysis was done on the primary tumor group using the karyometric features selected in the recurrent tumors. In both cases (line 1 and line 2) sub-optimal division of P and NP cases is obtained.

Tumor grade and stage were entered in the discriminant analysis in addition to the karyometric features. However, due to low *F*-values, tumor stage, grade, and changes in tumor grade were not selected in the stepwise analysis: i.e. there is no additional value of the classical features to the karyometric features for the prediction of tumor progression.

All recurrent tumors and 6 of 7 primary tumors with a 5cER higher than 10%, suggesting non-diploid tumor cells, showed progression during follow up (Figure 4).

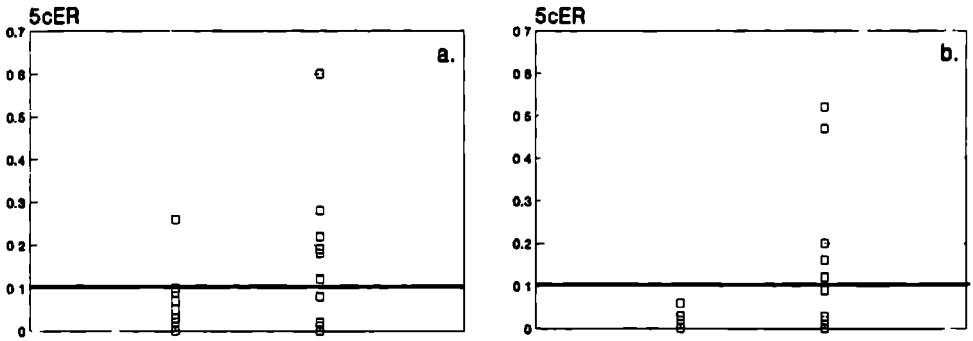

Figure 4. 5c Exceeding Rate in primary (a) and recurrent tumors (b). The mean value per case is plotted and the cases are divided into progressive and non-progressive disease during follow up.

DISCUSSION

Superficial transitional cell tumors of the urinary bladder are characterized by a tendency to recur after resection. Gilbert and associates (1978)[19] reported a recurrence rate of 70% and found an increase in tumor grade in 25% of the recurrent tumors. Other studies reported up to 90% recurrence rate[1] depending on grade, stage, and treatment modality used.[2-5,7,21]

From a prognostic point of view the risk of invasion of a treated primary superficial tumor is more important than recurrence rate. In patients with a recurrent tumor, 10-30% progression to invasion is detected.[8] Moreover, Althausen and associates[9] reported progression rates as high as 83% in patients with frequent tumor recurrences and multiple tumors. For superficial bladder tumors progression and recurrence rate are highest in pT1G3 tumors.[21] However, progression rates for pTaG3

and pT1G2 tumors are similar, indicating that tumor stage alone can not predict tumor progression accurately. The pathological staging results from the present study showed no difference in progression rate between all pTa and pT1 primary tumors. Tumor staging in small superficial bladder tumors is often difficult[13] and might have caused the discrepancy between these findings and earlier studies.

In case of a superficial bladder tumor, apart from tumor stage several tumor characteristics have been investigated for prediction of tumor behavior. Tumor grade is generally used as a stage-independent indicator of tumor behavior. The subjectivity of tumor grading has led to quantitative methods to describe tumor cells and nuclei in microscopic images.

In the present study quantitative light microscopy is used to characterize cell nuclei in bladder tumors. Since only a limited number of nuclei can be analyzed, selection criteria will influence results. Contrary to other studies[10,15] that used selected nuclei, in the present study only the tumor area was selected to reduce subjectivity. Subsequent analysis was performed in randomly chosen images within this area. This procedure was also used in an earlier study and resulted in good correlation between grading and karyometric features.[16]

Several nuclear features were significantly different between the progressive and non-progressive tumors in the primary tumor group. Primary tumors containing non-diploid cells reflected by high values for 2cDI and 5cER often progressed. Although Blomjous and associates (1989)[15] found correlation between the karyometric features of the primary tumor and progression rates, the present data showed best prediction of progression by karyometric features of the recurrent tumors. The presence of large nuclei in recurrent tumors, measured by the 90th percentile of the nuclear profile area was correlated with tumor progression. This is in agreement with earlier studies.[10,15] Blomjous and associates (1989)[15] found increased recurrence and progression rates in patients with tumor cells with large nuclei (mean profile area > 95μm^2). The presence of large nuclei correlated with tumor grade in a study by Ooms and associates (1983).[10] Unlike the present study, these histological studies[10,15] applied criteria for selection of nuclei based on nuclear size which may affect reproducibility.[23] Nuclear size, either measured in selected or in random chosen nuclei is clearly correlated with tumor behavior.

Nuclear polymorphism and abnormal nuclear shape are characteristics of high-grade tumors. Montironi and associates (1985)[24] could discriminate the different tumor grades using the nuclear roundness factor (NRF). For reasons discussed elsewhere[11]

the NRF is theoretically less useful in grading bladder tumors. Therefore we choose, besides the NRF, a shape feature derived from the Freeman chain code of the nuclear profile contour. This method enables detailed analysis of convexities and concavities in the nuclear structure.[18] Although the mean NRF was significantly lower in progressive tumors, indicating more abnormal nuclear shapes in these tumors, the standard deviation of the MAC, a Freeman chain code derived feature, had additional value to nuclear size for predicting progression and is thus preferable over the NRF.

The finding that features of the primary tumor were of less predictive value, using either the classical or karyometric features is somewhat disappointing and illustrates the importance of regular follow up of patients with superficial bladder tumors. In seven cases the classification of the primary tumor was different from that of the recurrent tumor (in two cases this meant 'down-grading': i.e. whereas the primary tumor predicted progression, the recurrent tumor did not). In none of the seven cases prediction based on the primary tumors was correct and only in four the change in karyometric prediction was accompanied by a change in grade or stage which illustrates the grade-independent prognostic value of karyometric analysis.

Although the discriminant function in primary and recurrent tumors is not equal, Figure 3 illustrates that the classification results do not improve when the function derived for the recurrent tumor is used to classify the primary tumors (see line 1 in Figure 3).

Soloway (1989)[25] divided recurrent tumors into 'true recurrences' and 'new occurrences'. Another possible cause of recurrence is tumor implantation.[25] Which cause of tumor recurrence underlays the recurrent tumors in the present study is difficult to determine based on karyometric features alone. To test the similarity of the two tumors, the differences of the karyometric feature values between primary and recurrent tumor were taken into analysis. Differences in karyometric features did not correlate with progression. Whereas these differences were of additional value to the primary tumor data for predicting progression no additional predictive value to the data from the recurrent tumors was shown. We therefore conclude that a change in tumor as measured with karyometric analysis can not predict progression and that it is the data from the recurrent tumor rather than from changes in the primary tumor that determine prognosis.

In conclusion, karyometric analysis of recurrent superficial bladder tumors has a strong predictive value for tumor progression. Multivariate analysis techniques indicate additional predictive value of the features that describe the presence of extremely

large (NINAR) and polymorph (SD MAC) nuclei. Visual grading of the histological slides has no additional value to these features. All karyometric features could be measured on routine formalin-fixed, paraffin-embedded material, and can easily be incorporated in routine diagnostic procedures. The low predictive value of analysis of the primary tumor, however, indicate the need for careful (karyometric) follow up of patients with superficial bladder tumors.

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Chapter 4

FOLLOW UP OF PATIENTS WITH SUPERFICIAL BLADDER CANCER BY KARYOMETRY OF BLADDER WASHINGS

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van der Poel, H.G. Oosterhof, G.O.N., Debruyne, F.M.J. and Schalken, J.A.: Image analysis in superficial transitional cell carcinoma of the bladder. Sem. Urol. 11:164-170, 1993.

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ABSTRACT

A quantitative cytological analysis of 1600 bladder wash samples from 695 patients attending operation room and outpatient department was performed. In a pilot study karyometric analysis was useful for the discrimination of tumor and non-tumor samples and a correlation of the karyometric features with the histological tumor grade was found. In a logistic regression analysis (n=115) for the presence of tumor, two karyometric features were found: the 2c Deviation Index and the mean of BEN, a nuclear shape feature. The function was evaluated in a large population (n=1336). Sensitivity of the karyometric analysis for the detection of tumor by bladder washing was 90.6% and specificity was 69.8%. The low specificity was possibly caused by the absence of histological confirmation in the outpatient samples where only cystoscopical interpretation was available: 97% of the false-positive samples were obtained at the outpatient department. Moreover, the recurrence rate during follow up (median 7.3 months, range 3-26.3) was 20% for the false-positive scored samples, whereas overall recurrence rate was 4.2%. The logistic evaluation showed that bladder washings fixed in Carbowax are well suited for mailing and karyometric analysis. This quantitative approach to cytology can be particularly useful for the longitudinal follow up of patients with superficial bladder cancer.

INTRODUCTION

The follow up of patients with superficial bladder cancer is done by cystoscopy and urine cytology. Low reproducibility and the inability of quantitation hamper visual interpretation of cytological material.[1] Flow-cytometric studies showed that ploidy analysis of bladder wash material is a sensitive method of tumor detection.[2-7] Since, flow cytometry does not enable simultaneous visual interpretation and selection of cells, quantitative light microscopic techniques can be used for DNA content analysis instead.[3,8] Moreover, the latter technique can also be used for the quantitation of a panel of nuclear and cellular features other than DNA content.[9] The group of Koss in the Montefiore Medical Centre in New York has extensive experience with an image analysis system for the classification of cells in voided urine.[1,10,11] Sensitivity of this system was particularly high for high-grade tumors but low-grade lesions were less readily diagnosed. The cell-to-cell reference classification and the use of voided urine instead of bladder wash material are two reasons for the low diagnostic yield. In this chapter we will discuss the use of a quantitative approach to cytology of bladder washings. Besides nuclear DNA content we analyzed several karyometric features on Feulgen-stained cytospin preparations to answer the following questions: 1. Can we develop an inexpensive system for the quantitative analysis of cytological material of bladder washings?; 2. Does karyometric analysis offer information on the histological tumor characteristics and can it be used to predict early tumor recurrences?; 3. Is a grading system based on the karyometric analysis clinically useful as a diagnostic tool?.

MATERIAL AND METHODS

Patient Population and Material Sampling

Patients attending outpatient department or operating room during follow up or treatment of a superficial bladder cancer were eligible. The material was obtained by rinsing the bladder vigorously, at least twice with saline, either through a catheter or a cystoscope. 25cc of the material was instantly fixed in 25cc 50% ethanol containing 2% polyethylene glycol (Carbowax) and stored at 5°C. Bladder washings were always performed after emptying of the bladder and prior to intravesical manipulations or instillations. After arrival in the laboratory the material was centrifugued (2000x, 10 min.) and the pellet resuspended in Carbowax fixative. For staining, the material was Cytospin centrifuged (Cytospin, Shandon) on gelatin-coated slides, post-fixed with

Böhm fixative (85% methanol, 10% buffered formalin, 5% acetyl acetic acid glacial) and Feulgen stained (5 N HCl 60 min., Schiff-reagens (Merck), 30 min. both at room temperature).

Karyometric analysis

The karyometric analysis was performed on the Feulgen stained slides using a PC-based image analysis system. The system consisted of a framegrabber board (VFG-framegrabber, Imaging Technology, Woburn, MA) in a Compaq 386s personal computer. Image handling and analysis software was written in TIM (TEA, Dordrecht). Images of 512x512 pixels were digitized and stored on computer hard-disk, prior to analysis. In the image analysis steps the images were corrected for background shading and filtered prior to segmentation applying local segmentation routines based on grey value histogram interpretation (iso-data threshold determination). After thresholding, several binary operations were conducted including detection and separation of overlapping objects. Of each nucleus a panel of morphometric, densitometric, and chromatin textural features calculated (Table 2 and 3, Chapter 1) and the automaticly contoured and numbered nuclei were marked in the image for verification by the technician. Of each slide 50 randomly selected images containing 100 to 500 nuclei were analyzed after recording on the harddisk of the computer. Recording of these images took 4 minutes per slide. The 50 images per slide were automaticly analyzed, without intervention by the technician, in 80-100 minutes. After the automatic analysis the selected nuclei are contoured and numbered in the images enabling 'post-analysis' verification of the objects visually. Lymphocytes in the material were used as reference for 2c DNA content after applying a correction factor of 1.19. For interpretation of the DNA histogram obtained by analysis of the integrated optical density (IOD) per nucleus, the 2c Deviation Index (2cDI), 5c Exceeding Rate (5cER), and a measure of polyploidy (PPR, percentage of polyploid nuclei) were calculated. Moreover, DNA histograms were scored as diploid, aneuploid, or tetraploid. Aneuploidy was defined as an abnormal peak outside 2c (1.8-2.2c) and 4c (3.8-4.2c) regions, tetraploid histograms had 4c peaks containing more than 20% of cells with or without G_2M peaks at 8c DNA content. Of each sample the mean value for each karyometric feature was calculated. As a measure of variation within the sample the difference between the 15th and 85th percentile was calculated.

Pilot analysis

Initially 124 bladder washings for a pilot analysis were sampled prior to histological biopsies of the tumor. Two karyometric features showed correlation with the presence of tumor: 2c Deviation Index (2cDI) and a nuclear shape feature PASS.

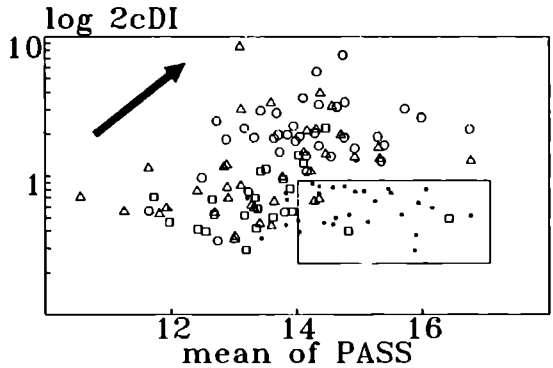

Figure 1. Scatterplot of 2cDI and mean PASS per sample by histological tumor grade (G1:square; G2:triangle; G3:circle). The box contains 85% of normal cases. The arrow indicates the increase in tumor grade (n=124).

Moreover, a correlation of the karyometric features with histological tumor grade was found (Figure 1). These findings suggested the applicability of the system for the grading of bladder wash samples. After adaptation of the image analysis facilities (extension of image storage capacity and faster processing), a study for validation of these results was conducted.

Hence, 1600 samples were obtained in five urological institutes (october 1990 to december 1992) from 695 patients. Each bladder washing was send to our laboratory and accompanied with an application form with patient data. From the 1600 samples 149 (9.3%) were not suitable for karyometric analysis due to an abundant number of inflammatory cells or low number of urothelial cells.

For calculation of the multivariate analysis function, 115 samples were analyzed in a logistic regression analysis. The samples were selected from the entire population on the following criteria: the sample should be the first sample of the patient present in the study and histopathological confirmation of the presence or absence of tumor should be available. The group was divided into samples from patients with (n=88) and without (n=27) histological cancer.

To validate the function, the chance on tumor, as calculated from the karyometric features was correlated with the pathological or cystoscopical findings in a large population (n=1336). The material was divided into different groups: samples without macroscopical or microscopical tumor (n=830); samples with tumor (n=203); samples taken during intravesical therapy (n=155); samples with non-transitional cell cancer lesions, like squamous cell carcinoma (n=8); and samples from patients with prostatism complaints (n=140). In 111 cases the sample was obtained at the operation room prior to transurethral resection of the tumor, 1225 samples were from patients attending the outpatient department. The distribution of samples per patient is given in Table 1.

Table 1. Distribution of number of samples per patient.

number of samples per patnt:	1	2	3	4	5	6	7	8	9	10	11	12	total
number of samples:	695	280	153	101	80	55	36	25	14	7	4	2	1451
number of patients:	415	127	46	27	25	19	11	11	7	3	2	2	695

Patient Data Management

Since for each bladder wash sample an application form was filled in all data concerning earlier treatments were available at the time of the karyometric analysis. The databases containing this information were linked to the karyometric analysis so that the report form contained both karyometric analysis results of the analyzed and prior samples as well as all data available of the patient.

Reproducibility

In Chapter 2 the reproducibility of the karyometric analysis among three technicians was tested. It was concluded that at least 100 nuclei should be analyzed when random selection is applied. Since samples were send by mail we also tested the influence of fixation time and transportation. When fixed in Carbowax solution, fixation of at least three days is necessary to obtain optimal Feulgen staining. The samples obtained in the collaborating institutes were compared with samples in our institute. The former samples were send by mail whereas the latter were received in our laboratory within one day after bladder rinsing. Karyometric feature values for samples without tumor were compared and no significant difference between samples send by mail and the

samples from our institute was observed ($P > 0.10$, ANOVA).

Statistical Analysis

To obtain a variance stabilizing transformation the logarithmic value of the karyometric features was used (see Chapter 1). For comparison of two groups the non-parametric U test analysis according to Mann-Whitney was applied. A multivariate logistic regression analysis of the karyometric features was performed to discriminate the tumor and non-tumor group. To avoid patient dependent influences, only the first sample per patient was used to calculate the regression function.

RESULTS

Of the sampled 1451 bladder washings, follow up was available with a median of 7.3 months (range 3 - 26.3 months). In the univariate analysis of the 115 samples from the operation room, several karyometric features were significantly different between tumor and non-tumor samples. Of the nuclear shape features mean BEN per sample was significantly higher ($P < 0.0001$, Mann-Whitney U test), whereas mean PASS was significantly lower ($P<0.0001$, Mann-Whitney U test) in tumor samples compared to non-tumor samples (Figure 2 and 3). A high negative correlation for mean PASS and mean BEN per sample was found ($r = -0.8531$, $P < 0.001$).

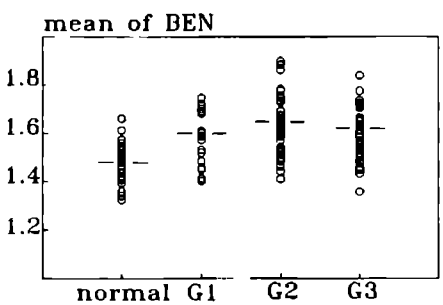

Figure 2. Mean BEN values by histological tumor grade.

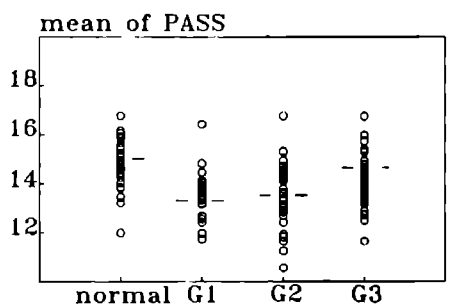

Figure 3. Mean PASS values by histological tumor grade.

Nuclear size as estimated by the nuclear profile area increased with an increase in histological tumor grade and was significantly higher in grade-3 compared to grade-1

and 2 tumors ($P<0.0001$, Mann-Whitney U test). The analysis of nuclear DNA content revealed significant higher values for 2cDI, 5cER, and PPR in samples from patients with bladder cancer. The 2cDI and 5cER increased with tumor grade.

In the multivariate analysis of the karyometric features, the mean BEN and 2cDI were selected in the model for the prediction of the presence of tumor (Table 2, Figure 4).

Table 2. Regression function calculated in the logistic regression analysis of the karyometric features for the presence of tumor.

	ß	$SE_ß$	*P*
intercept	-9.2864	2.0972	0.0001
log(BEN)	25.6120	5.3981	0.0001
log(2cDI)	1.2261	0.5431	0.0240

$$XBeta = -9.2864 + (25.6120 * \log(BEN)) + (1.2261 * \log(2cDI))$$

and:

$$P_{tumor} = \frac{e^{XBeta}}{1 + e^{XBeta}}$$

The lines for the chances on tumor (p) can be obtained as follows:

$$di = \left(\frac{e^{9.2864}}{BEN^{25.6120} \times (1/p - 1)} \right)^{1/1.2261}$$

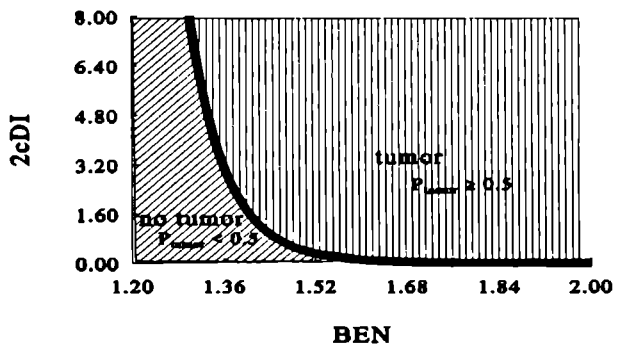

Figure 4. Distribution of tumor and non-tumor scores in the scatterplot of the 2cDI and the mean BEN per sample. Note the logarithmic character of the function, due to the use of logarithmic karyometric features in the analysis.

The distribution of the samples in this population is given in Table 3. Sensitivity of the karyometric analysis for the detection of tumor in the bladder was 90% in this population; specificity 70%. All eight false-positive scored samples ($P_{tumor} \geq 50\%$ and no histological tumor) had P_{tumor} values smaller than 75% (Figure 5). Of the seven false negative scored cases ($P_{tumor} < 50\%$ but histologically tumor present) six were histologically grade-1 and one grade-2 tumors. All grade-3 and CIS cancers had $P_{tumor} > 50\%$. The sensitivity for grade-1 tumors was 71% (15/21) and 96% (29/30) for grade-2 cancer (Figure 6).

Table 3. Cross table of the karyometric prediction of tumor and the histological findings in the 115 samples analyzed in the logistic regression analysis (n=115).

	karyometric prediction no-tumor ($P_{tumor} < 50\%$)	tumor ($P_{tumor} \geq 50\%$)
actual status		
no tumor	19	8
tumor	7	81

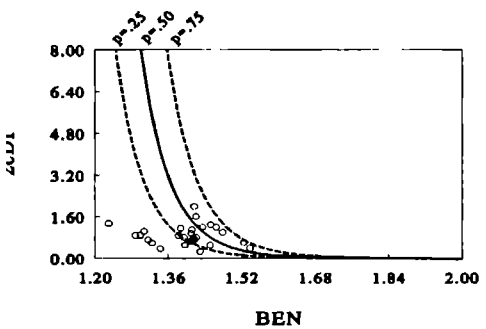

Figure 5. Scattergram of samples without histological bladder cancer (n=27).

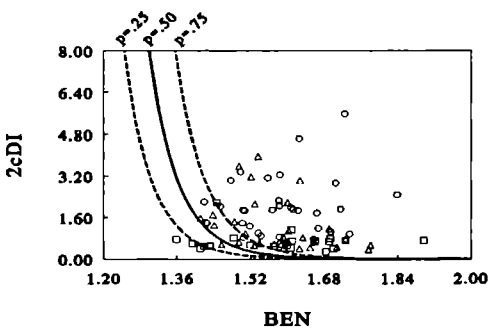

Figure 6. Samples with histological cancer by tumor grade (n=88) (square:G1; triangle:G2; circle:G3).

Verification analysis

To evaluate the function found in the logistic regression analysis for each of 1033 samples the P_{tumor} was calculated and each sample was scored as tumor ($P_{tumor} \geq 50\%$)

or non-tumor (P_{tumor} < 50%). In Table 4 the karyometric score is compared to the actual status. Sensitivity for the detection of tumor in this population was 90.6 % and specificity 69.8%. Of the false positives (n=250) 242 (97%) were samples obtained at the outpatient department, whereas overall 81% was obtained at this location (P < 0.0001, chi-square test).

Table 4. Cross table of actual status (assessed histopathologically or cystoscopically) and karyometric prediction of the presence of tumor (n=1033)

	karyometric prediction	
	no-tumor (P_{tumor} < 50%)	tumor (P_{tumor} ≥ 50%)
actual status		
no tumor	580	250
tumor	19	184

Moreover, follow-up data in the false positive group revealed a significant higher frequency of recurrent tumors compared to the rest of the population (20% versus 4.2%, P < 0.0001, chi-square test). Of all samples followed by a tumor recurrence during follow up (n=51), 44 (86.4%) showed a karyometric score suspicious for tumor (P_{tumor} ≥ 50%) (Figure 7).

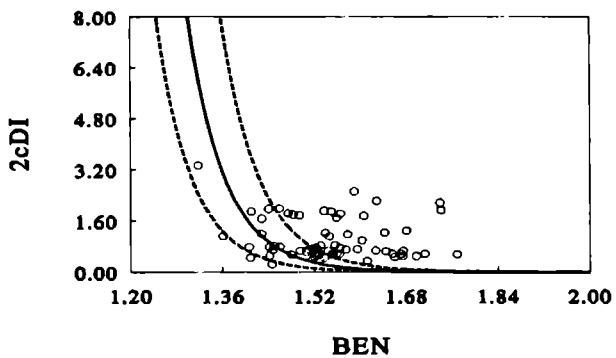

Figure 7. Scattergram of karyometric features BEN and 2cDI for samples followed by tumor recurrence (n=51). Note that only seven cases had P_{tumor} values smaller than 50%.

Cytology and karyometric analysis

To compare the sensitivity and specificity of the karyometric analysis with the visual cytology, a subset of 104 samples was analyzed with both techniques. Due to inadequate material (too few diagnostic cells) 8 samples were not suited for cytological analysis. In Table 5 the cross tables for the cytological and karyometric analysis are given. Sensitivity for cytology was 71.6% and for the karyometric analysis 95.2% in this population; specificity was 60% and 65% for both techniques, respectively. Of the false negative samples in cytology, 43% (10/23) were tumors with a histological grade higher than grade 1. The false negatives in the karyometric analysis consisted of three grade-1 and one grade-2 tumor.

Table 5. Comparison cytological and karyometric analysis with actual tumor status (n=104).

	cytology		karyometric score	
	no tumor	tumor	no tumor	tumor
actual status				
no tumor	9	6	13	7
tumor	23	58	4	80

Bladder washing and voided urine

Since the bladder washing procedure still requires invasion with either a catheter or a cystoscope into the bladder, we compared it with the karyometric analysis of voided urine as well. In the Figures 8 and 9 the correlation between the karyometric analysis of voided urine sample and subsequent bladder washing is shown for the two karyometric features in the logistic regression function (n=40). No significant correlation was found for the two features. In a multivariate analysis for the discrimination of tumor versus no tumor samples, only the karyometric feature values from the bladder wash samples were of value.

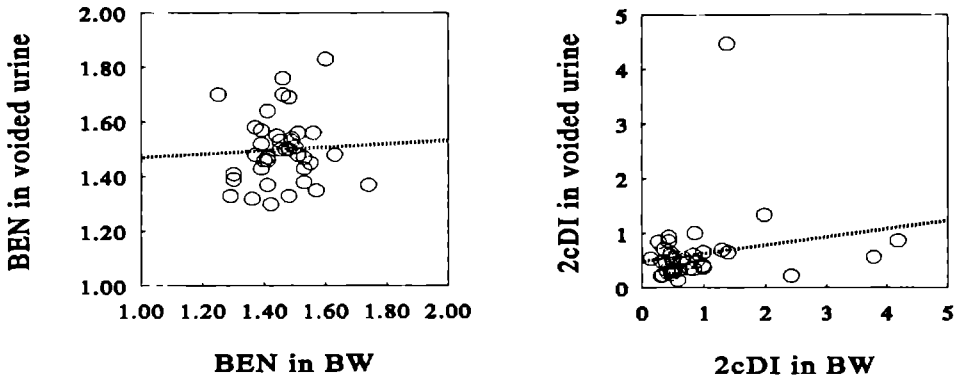

Figure 8 and 9. Correlation of karyometric features mean BEN and 2cDI in bladder washing and concomitant voided urine sample.

DISCUSSION

Superficial transitional cell carcinoma (TCC) of the bladder has a high recurrence rate after transurethral resection (30-90%).[12,13] Grade, prior recurrence rate, and multiplicity are the most important predictors of recurrences in these tumors.[14,15] Compared to superficial cancer, invasive disease constitutes a much greater threat to the patient's health. Because 10-30% of superficial TCC progress to an invasive phenotype, prediction of tumor progression forms an important issue in bladder cancer management.[16,17] Progression to an invasive phenotype is best predicted by; the tumor grade, size, and recurrence rate.[14] Grade-3 tumors were five times more prone to metastasis than grade-1 tumors (30% vs 6%).[14] These findings indicate the importance of tumor grading as a prognostic factor.

Exfoliation of tumor cells in urine, as was initially demonstrated by Papanicolaou in 1945,[18] offers a powerful diagnostic tool for detection and grading of patients with TCC of the bladder. Although sensitivity for high-grade tumors is high (94-100%), low-grade lesions are less readily detected by cytology (sensitivity: 30-60%).[19,20] The criteria for detection of neoplastic cells in urine are seemingly straight forward, but the sometimes low number of abnormal cells, the inability of quantitation, and the high variety of atypical characteristics demand much experience of the cytologist. Reproducibility of cytological grading systems, therefore, is disturbingly low.[1]

Furthermore, comparison of subsequential samples from one patient for the early detection of cytological abnormalities is arduous.

Quantitative analysis of cytology can be divided into flow cytometric and image analysis methods.

Flow cytometric studies showed the diagnostic value of DNA content analysis of bladder washings. Sensitivity for the detection of tumor varied from to 50 to 83%, dependent on tumor grade.[2,3,4,6,7,21] DNA content analysis provided information on tumor behaviour: of diploid superficial bladder tumors 2-8% progress to invasive disease (≥ T2), whereas 35-50% of aneuploid tumors do. Although not conclusive, these data show that DNA content analysis is an important prognostic parameter. A disadvantage of flow cytometry is the fact that visual control of measured objects is impossible. Visual control is particularly useful in samples with much debris and large amounts of lymphocytes. Koss et al. (1989)[3] found DNA content analysis by image analysis techniques to add information to flow cytometry by more selective analysis of abnormal cells as is made possible by image analysis techniques. In a recent study Amberson et al. (1993)[6] applied DNA content analysis by image analysis in those cases where cytological interpretation was ambiguous. Sensitivity for both low-grade and high-grade lesions increased with more than 10%. DNA content assessment by image analysis, therefore, is an important diagnostic tool in quantitative cytology.

Since visual tumor grading is based on several cellular and nuclear features, attempts have been made to come to multivariate quantitative grading by image analysis of voided urine sediments.[10,22] Bladder washings enable the harvest of more and better preserved material than voided urine.[4,23] We, therefore, tested a multivariate approach for the early detection and grading of recurrent bladder tumors using bladder washings. The features were all based on nuclear characteristics applied in the visual assessment of tumor grade. In Chapter 1 we described the use of nuclear size in a cytomorphometric study and found it to be correlated with visual tumor grade. Besides size, nuclear shape and chromatin texture features were included in the present study. We calculated nuclear shape features based on the Freeman chain code.[24,25] In this way size independent measures of nuclear shape were obtained. A correlation with histological tumor grade was observed. Low-grade tumors were characterized by low 2cDI values often in normal range. Detection of these tumors was frequently possible due to abnormal values of nuclear shape. For high-tumors abnormal DNA ploidy patterns resulted in increased 2cDI values, whereas nuclear shape was also different from tumor free samples. In the pilot study, a nuclear shape

feature (MPASS) describing the degree of ellipse-shape of the nucleus was found to be particularly of value for the detection of low-grade cancer. Since the 2cDI of low-grade tumors was in normal range an additional value of this nuclear shape feature was expected. In a multivariate analysis the 2cDI and MBEN, a feature highly correlated with MPASS, were selected for the prediction of tumor. The value of karyometric analysis was confirmed in a large population. Of the grade-1 tumors 71% could be detected, which compares favorably with the detection rate of low-grade low-stage lesions by flow cytometry (64%)[2] and cytology were low-grade lesions are identified infrequently (51%).[19,26] Sensitivity for histologically high-grade lesions was 100% for the karyometric analysis and comparable to detection rates of flow cytometric analysis.[3]

These data show that karyometry is well suited for the detection of bladder cancer by bladder washings. Although a difference between the population in the pilot study and the verification analysis existed (in the pilot study, histological confirmation of tumor was present; in the second analysis tumor was only detected by cystoscopy) in both populations a tumor could be missed, resulting in a 'false'-positive karyometric score. This is one of the reasons for the low specificity in both populations.

Another cause of the relatively low specificity could be the predictive value of the karyometric analysis. Tumor recurrence and progression are determine prognosis in superficial bladder cancer.[14] Tumor markers predicting recurrence and progression, therefore, are subject of many studies. Proliferation rate, ploidy pattern, and several possible molecular biological markers and monoclonal antibodies are of prognostic value in bladder cancer. The multifocal origin of bladder cancer suggests a general disease of the urothelial mucosa. Tumor biopsies alone do not provide information on the general status of the mucosa. Bladder washing provides a simple means for sampling material from the entire bladder mucosa. Compared to voided urine more and better preserved cells are obtained, originating from the entire bladder mucosa.[27] The short-term follow up in the present study revealed a high recurrence rate (20%) in patients with negative cystoscopical findings but a karyometric score that indicated tumor. Moreover, 86% of all tumor recurrences were preceded by a cystoscopically tumor-negative sample with positive karyometry score. The low specificity of the karyometric analysis therefore, is partly caused by its predictive value of later tumor development. Hence, careful follow up of the patients with a false-positive karyometric score is advisable. We therefore developed a report form presenting both karyometric score and clinical history (Figure 10).

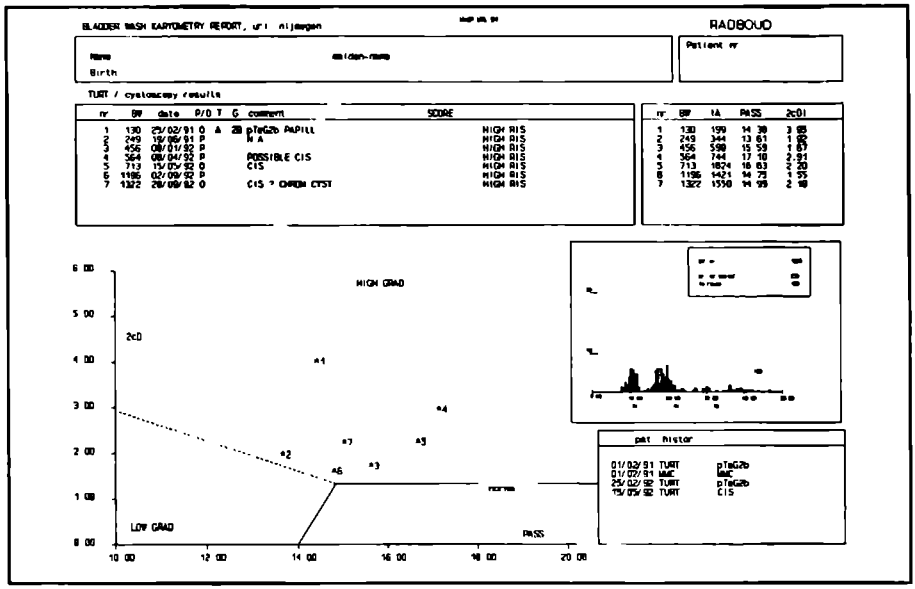

Figure 10. Report form with karyometric analysis results and patient data.

Since theoretically "normal" and "abnormal" cells are present concomitantly in the sample, in an earlier study we also investigated the distribution of cells with different karyometric phenotype within the samples.[28] Based on the karyometric score per sample each nucleus was scored as either "normal"', or "abnormal" depending on its ploidy level (deviation from 2c in the population) and shape as calculated by BEN. For each sample we then calculated the percentage of normal nuclei, i.e. nuclei with 2cDI and BEN values within normal range. The percentage of normal cells was significantly lower in tumor samples ($P < 0.05$). Moreover, with an increase in tumor grade, the percentage of normal cells decreased. This may be caused by the higher exfoliation rate of high-grade tumor cells. Unfortunately, tumor size could not be established accurately in these patients but will certainly influence the number of "abnormal" nuclei shed in the bladder washing. Surprisingly, however, percentages for "normal" nuclei were not significantly different in invasive and superficial tumors.

The influence of intravesical therapeutic instillations on (bladder wash) cytology have been described.[30,31] BCG instillations resulted in an increased number of polyploid cells[2] whereas mitomycin-C (MMC) treatment gave rise to cytological

abnormalities several months after instillation.[30-32] The bladder wash samples taken during or within 2 months after intravesical therapy were separatly analyzed. The percentage of recurrent tumors (5%) during follow up in this group (n=155) was similar to the entire population. The patients in the intravesical instillation group that developed a recurrent tumor had P_{tumor} values larger than 70%. These findings indicate that, although, the karyometric score is influenced by BCG or MMC instillations, samples of patients at risk still exhibit more abnormal karyometric scores.

The logistic evalution shows that the applied combination of fixation and mailing of samples provides adequate sample preservation for cytological as well as karyometric analysis. Recently the NCI provided recommendations for the preservation of bladder washings for flow cytometry.[4] We added polyethylene glycol to the described fixation method in 50% ethanol for better preservation of cytological features.[29] For storage longer than 7 days the material was centrifuged and resuspended in Carbowax and stored at -20°C.

From our findings we conclude that karyometric analysis provides a sensitive method for the detection of tumor in the bladder. A karyometric score of two nuclear features (2cDI and BEN) was designed. High-grade lesions were always detected whereas sensitivity for low-grade lesions was 71%. The low specificity (58%) is partly explained by the predictive value of later tumor recurrences.

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Chapter 5

PROGNOSTIC VALUE OF KARYOMETRIC AND CLINICAL CHARACTERISTICS IN RENAL CELL CARCINOMA: QUANTITATIVE ASSESSMENT OF TUMOR HETEROGENEITY

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ABSTRACT

The variation in tumor cell differentiation within one renal cell carcinoma, also termed tumor heterogeneity, renders visual tumor grading of these carcinomas difficult. Karyometric analysis enables description of nuclear characteristics of multiple tumor areas. Hence, karyometric analysis can be used to quantify tumor heterogeneity and thus may aid in a more objective grading of renal cell carcinoma. Of 121 patients with renal cell carcinoma (RCC) (tumors UICC stages: 1 [5 cases], 2 [23 cases], 3 [33 cases], and 4 [60 cases]) clinical and karyometric features were studied to obtain routinely applicable prognostic factors. Several parts of the tumor were analyzed, to obtain a measure of tumor heterogeneity. Univariate and multivariate Cox regression analyses were used to determine the predictive value of karyometric features independent of tumor stage and other clinical characteristics. The Cox univariate regression analysis showed correlation of several clinical and karyometric characteristics with survival. Of the clinical characteristics TNM stage, tumor size, weight reduction and performance status were significantly associated with survival. The karyometric features, especially those measurements associated with tumor heterogeneity (e.g. differences in nuclear size or chromatin texture between tumor subpopulations) were of value in predicting prognosis. In the Cox multivariate regression analysis the Robson and UICC-stages proved to be the most powerful predictors of survival ($P<0.0001$). Of the clinical features, weight reduction and performance score were the only characteristics offering additional information to tumor stage ($P<0.0001$). From the karyometric analysis quantification of anisokaryosis in the tumor at time of diagnosis offered additional prognostic information. Moreover, the differences of karyometric features within the tumor presumably associated with tumor heterogeneity correlated with survival. Using the features from the multivariate analysis, prognostic groups could be defined. We conclude that karyometric analysis offers a useful means for quantifying tumor heterogeneity. Multivariate Cox analysis revealed additional value of a grading system based on karyometric analysis to tumor stage.

INTRODUCTION

Renal cell carcinoma (RCC) accounts for 3 percent of all malignancies and the 5-years survival rate is 30 to 60%.[1] The tumor is characterized by its unpredictable clinical course and only limited effective treatment modalities are available. The staging system initiated by Flocks and Kadesky and popularized by Robson and associates is based on the extent of the tumor and correlates with survival.[2,3] Another widely used staging model is the TNM system of the American Joint Committee for cancer staging which classifies the tumor according to the anatomical extent of disease.[4] This has been the basis of the staging by the International Union against Cancer.[5] However, within subgroups of patients as defined by these classification models, the clinical course of RCC is still diverse.

No important improvement in the survival of patients with RCC has been achieved during the last decades. Surgery remains the cornerstone of treatment and the only way to cure the patient. New treatment modalities like immuno- and chemotherapy are under investigation.[6,7] Response rates for these treatments in the overall population of metastasized disease are low (10-30%). There are, however, indications that selection of patients based on prognostic factors can improve response rates.[8,9] Therefore,we investigated clinical and pathological features of patients with RCC for their prognostic value.

For several tumors, grading offers prognostic information. Considering the extensive tumor heterogeneity in renal cell carcinoma[10] we sought to develop an objective grading system analyzing several parts of the tumor. Karyometry is used to quantify nuclear features and offers an objective measure of tumor phenotypic characteristics. Since heterogeneity will probably result in nuclear differences in light microscopy, comparison of the karyometric feature values of different areas within the tumor can be an objective measure of tumor heterogeneity.

In order to obtain insight in the prognostic value of such karyometric grading the features were correlated with patient survival. Moreover, the karyometric grading was compared to classical prognostic features in a multivariate analysis.

MATERIAL AND METHODS

Material

Data were obtained of 121 patients (77 men; 44 women) with a RCC treated between 1983 and 1990 with tumor nephrectomy. The patient age ranged from 32 to 85 years (median 67 years). The median follow up was 21 months (range 4 - 133 months).

All the patients could be classified according to the TNM(V)-model and staged according to the Robson and the International Union against Cancer (UICC) classification models.[3,5]

For patients with metastatic disease several adjuvant treatments were given. In 65 M_+ patients one or more forms of immuno- and/or chemotherapy were given (IFNalpha + IFNgamma: n=58, IFNalpha: n=5, vinblastin: n=1, IL2 + IFNalpha: n=1). In 7 patients palliative radiotherapy was given after the tumor nephrectomy. At the time of diagnosis of the primary tumor these additional treatments did not improve survival significantly: 3 years survival rates for treated and non-treated metastasized disease was 0.27 and 0.30 respectively (log-rank test, $P>0.05$). Hence, all 121 patients could be grouped and analyzed for prognostic factors irrespective of the additional treatment given after tumor nephrectomy. We should notice that these therapy modalities were only used in patients with detectable metastases.

Clinical features

Various clinical characteristics of the patients, in earlier studies found to be associated with the outcome of the disease, were recorded for subsequent analysis. Besides patient age and gender, these included variables used for the classification models like pathologic stage, haematogeneous and lymphogeneous metastases, venal invasion of the tumor and tumor size. Moreover, general indicators of the patients physical condition were documented like performance status (Karnofsky score), history of weight reduction, and serum haemoglobin concentration at time of diagnosis.

Karyometry features

All paraffin-embedded archival material for each patient was reviewed by one pathologist (H.S.). Morphologically different tumor areas were marked for further karyometric analysis. The selection of areas was based on differences in histology (papillary and non-papillary) or cytology (clear, granular, and spindle cell). Karyometric analysis consisted of nuclear morphometry, densitometry, and chromatin

pattern analysis with a PC-based image analysis system (VFG-framegrabber board, Imaging Technology, Woburn, MA) in a Compaq 386s personal computer). Image handling and analysis software was written in TIM (TEA, Dordrecht). For analysis, 4 μm thick paraffin-embedded sections were deparaffinized and Feulgen-Schiff stained as room temperature. Within the marked areas, nuclei in 50 randomly chosen images were analyzed with a 100x objective. After shading correction and image filtering a local segmentation procedure was performed based on the grey-value histogram in the subimage. Of each nucleus in the recorded images a panel of karyometric features was measured (see Table 2 and 3 Chapter 1). At least 100 tumor nuclei were measured per tumor area. A sequence of 200 images could be analyzed in 5 hours. Of the analyzed nuclei data and images were recorded, thus enabling manual rejection of out-of-focus or faulty segmented objects after the measurement by the operator. For each sample the 2c Deviation Index (2cDI) and 5c Exceeding Rate (5cER) were calculated.[11]

Since in all tumors morphologically different tumor areas were marked by the pathologist, a measure of heterogeneity could be calculated as follows. In all tumors for every nuclear feature the tumor area with the highest and lowest value and their differences were determined. We also analyzed whether karyometric analysis of tumor areas marked by the pathologist was different from at random measurements in the tumor. Hence, besides data of the different tumor areas all values of the tumor areas were merged and median and difference of 15th and 85th percentiles were calculated to obtain a subpopulation independent measure of tumor heterogeneity.

The karyometric measurements were tested for inter-individual reproducibility among three technicians. For this purpose we have chosen randomly 28 out of the 121 RCC patients. Three technicians performed independently karyometric measurements of nuclei in the marked tumor areas. The measured feature values were analyzed for their inter-observer agreement using a two-way ANOVA. To test the reproducibility of the selection of tumor areas by the pathologist the procedure was performed twice by two persons in 10 randomly selected patients.

Statistical methods

The time from treatment to death was studied using survival analysis. The Kaplan-Meier method was used to estimate the survival function in a group. Differences of survival functions between groups were tested for statistical significance using log-rank test and Wilcoxon test. The 3-years survival rates with 95% confidence intervals were

calculated using the Kaplan-Meier method. Cox univariate and multivariate regression analyses (i.e. proportional hazard model) were performed to estimate the influence of the clinical and karyometric on the survival time.[12] Both forward and backward stepwise-selection procedures were used to find the best model in predicting the survival time. The stepwise-selection procedure was performed in two parts. In part I, the selection was used to find all clinical features to define either the best or an equivalent model. The Bonferroni correction was used for model entry of a variable. The same procedure was also performed on the karyometric feature group. In part II the selection procedure was performed on the features from the two feature groups selected in the previous step.

To obtain a prognostic score (RCC-score: good, intermediate, and poor prognosis) based on the selected features in the multivariate analysis, the linear part of the best multivariate model (XBeta) from the selection procedure was used. The levels of the RCC-score were arbitrarily chosen from jumps in the XBeta values of the present patients group.

RESULTS

Clinical features

During the follow-up period 68 (56%) patients died from RCC. The overall 3-years survival was 48%. The Kaplan-Meier estimators for survival for both Robson and UICC classification models in our patients are shown in Figure 1 and 2 respectively. The differences between the 3-years survival percentages of the stages is presented in Table 1.

Table 1. Three-years survival values for different stages in UICC and Robson stages.

	n	3-yrs	LCL	UCL
Robson stage				
A	20	0.83	0.60	1.00
B	14	0.77	0.55	1.00
C	32	0.53	0.35	0.71
D	55	0.27	0.15	0.39
UICC stage				
1	5	0.67	0.13	1.00
2	23	0.82	0.67	1.00
3	33	0.59	0.41	0.76
4	60	0.29	0.17	0.41

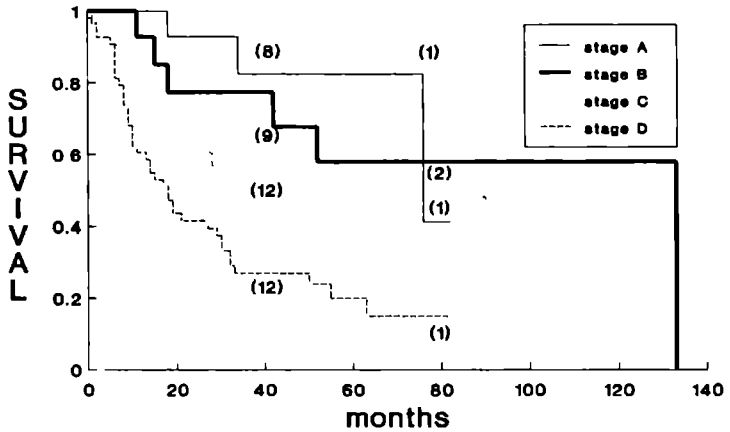

Figure 1. Kaplan-Meier survival curves for the Robson staging (stage A: n=20; stage B: n=14; stage C: n=32; stage D: n=55).

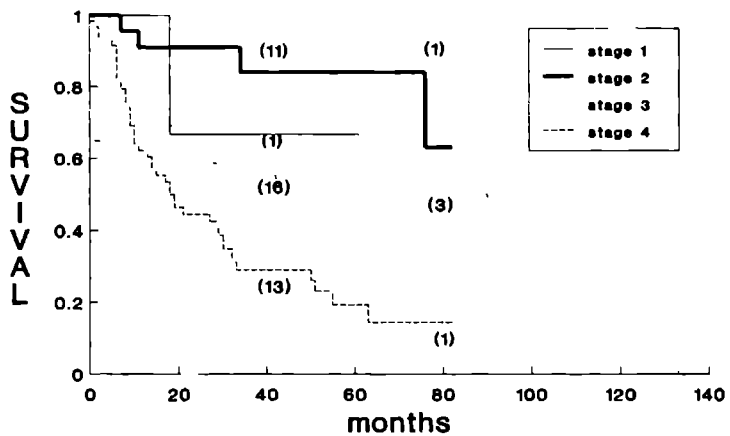

Figure 2. Kaplan-Meier survival curves for the tumor staging according to the UICC (1987) (stage 1: n=5; stage 2: n=23; stage 3: n=33; stage 4: n=60).

Several clinical features appeared to be related with survival. Of the pathological features an increase in T, M, or N stage was significantly correlated with a shorter survival ($P<0.005$, Cox regression analysis). The presence of venal invasion (V), patient age and sex, a low serum haemoglobin concentration at time of diagnosis, and the histological tumor type, however, were not significantly related with a shorter survival ($P>0.10$, Cox regression analysis). Other clinical characteristics that predicted a shorter survival were large tumor volume, a history of weight reduction, and a low Karnofsky score (Table 2).

Table 2. Results from the univariate Cox's regression analysis of the influence of the clinical variables at the beginning of the treatment on the survival in renal cell carcinoma.

	n	ß	$SE_ß$	*P*	relative hazard*
UICC-stage (1987)	121	-0.830	0.194	0.000	0.436
T	121	-0.530	0.175	0.002	0.588
N	121	-0.848	0.250	0.000	0.428
M	121	-1.230	0.266	0.000	0.292
V	121	-0.531	0.250	0.120	0.588
tumor size	106	-0.068	0.030	0.025	0.934
tumor type	121	-0.093	0.333	0.779	0.911
haemoglobin	112	0.203	0.105	0.053	1.225
weight reduction	112	0.806	0.260	0.002	2.240
Karnofsky score	108	0.046	0.013	0.000	1.047
age	121	-0.005	0.009	0.561	0.994
sex	121	0.039	0.257	0.878	1.040

*) relative hazard of survival per unit of measurement. Example: The probability to survive another month for a person is 7% lower in comparison to a person with a one mm smaller tumor.

Karyometry features

The reproducibility test of the marking of morphologically different tumor areas by the pathologist revealed differences in areas in 5 of 10 selected patients. Microscopic review of the different selected areas, however showed similarity in morphology. Moreover, the karyometric analysis of the areas resulted in non-significant differences. The karyometric measurements were tested for inter-individual reproducibility among three technicians. For only three of 32 karyometric features discrepancies were found among the technicians: the same observer scored significantly higher values for one feature (MH3) and differences were found for two other features (MAREA, MIOD).

This resulted in a higher percentage of explained variance due to the observers compared to the total variance (2%, 6%, 8% respectively). For the other features the explained variance was less the 1%. Comparison with the percentage explained by the variation between slides showed that differences between observers might be of minor importance: percentages explained by the variation between slides was 85%, 71%, and 68% respectively for the features (MH3, MAREA, MIOD) and 78% to 93% for the remaining features.

The mean number of morphologically different tumor areas as marked by the pathologist per tumor was 2.05 (range 1 to 5) and was not correlated with survival ($P>0.10$, Cox regression analysis).

Univariate analysis of the karyometric features showed a correlation between several nuclear characteristics and survival (Table 3). Of the nineteen karyometric features that were related with survival, two were subpopulation independent features. Eleven of the subpopulation dependent features that correlated with survival were features describing differences between tumor areas within the tumor. Karyometric features that showed best correlation with survival were variations in nuclear shape as described by the standard deviation of the bending energy (SDBEN), variances in nuclear size (SDAREA) and 'coarseness' of the chromatin as measured by the inverse difference moment of the requantitated pixel value co-occurrence matrix (Markovian feature H3).[13,28]

Multivariate analysis

Table 4a shows the results of the Cox multivariate regression analysis. Within this analysis the clinical variables tumor UICC-stage, the presence of weight reduction at time of diagnosis, and the Karnofsky score were the most important characteristics for survival. Besides these clinical characteristics the karyometric feature variation of the standard deviation of the nuclear size in different areas of the tumor showed additional prognostic significance. To study the significance of karyometric analysis in high-stage patients multivariate Cox analysis was performed on all UICC-stage 4 patients with known karyometric analysis (n = 52). Similar to the findings in the entire group, the karyometric analysis offered prognostic value as did the clinical characteristics. However, in the high-stage tumor group the highest value for the karyometric texture feature describing chromatin pattern coarseness per tumor (H3, inverse difference moment) was the best predictor of survival of both the karyometric and clinical features.

Table 3. Univariate regression analysis according Cox of karyometric features and survival in renal cell carcinoma.

feature	n	ß	$SE_ß$	*P*	relative hazard
subpopulation-dependent					
L MPASS	115	-0.145	0.067	0.029	0.865
L SDOD	115	-8.859	4.035	0.028	0.000
U MAREA	115	0.034	0.017	0.044	1.035
U SDIOD	115	0.113	0.040	0.004	1.120
U MH3	115	2.925	0.885	0.002	18.636
D 2cDI	115	0.060	0.028	0.029	1.063
D 5cER	115	0.040	0.020	0.043	1.042
D MAREA	115	0.016	0.008	0.046	1.016
D SDAREA	115	0.044	0.016	0.006	1.045
D MFPE	115	1.563	5.143	0.040	4.772
D MBEN	115	0.915	0.449	0.042	2.494
D SDBEN	115	3.741	1.693	0.005	42.149
D SDOD	115	3.910	3.808	0.009	49.924
D MIOD	115	0.058	0.025	0.020	1.060
D SDIOD	115	0.112	0.045	0.014	1.118
D MH3	115	3.969	2.055	0.016	52.942
subpopulation-indcpendent					
S IOD	115	0.137	0.055	0.013	1.147
MED H4	115	-1.187	0.599	0.048	0.305

L : lowest mean population value
U : highest mean population value
D : (D = U - L) diff. between values of highest and lowest population
S : diff. between 15th and 85th percentile of merged populations
MED : median value of all populations merged
2cDI = 2c Deviation index
5cER = 5c Exceeding rate
AREA = nuclear profile area
BEN = bending measure: difference in maximal and minimal value in smoothed difference Freeman chain code
FPE = form PE
H3 = Markovian texture feature: inverse difference moment
H4 = Markovian texture feature: rotation moment
IOD = integrated optical density
OD = optical density
PASS = nuclear shape feature: number of passes through threshold of smoothed difference Freeman chain code

The presence of weight reduction at diagnosis offered only additional prognostic value to this karyometric feature. This indicates that karyometric analysis was the most important prognostic factor is this group.

With the best predictors for survival, both the clinical (tumor stage, weight reduction, and Karnofsky score) and karyometric features (variation in nuclear size) we were able to form prognostic groups in the entire population of 121 patients. The results are shown in Table 4b and Figure 3. The prognostic score (RCC-score) was calculated using the formula found in the Cox regression analysis. Subdivision of the patients group into three groups based on the XBeta value resulted in significantly different Kaplan-Meier curves (Figure 3). Good prognosis (3-years survival: 80%) was found for patients (n=39) with a XBeta value lower than -2.3, whereas patients (n=28) with a XBeta value higher than -1.0 had a poor prognosis (3-years survival 21%). The 3-years survival for the patients with intermediate prognosis was 41% (n=54).

Table 4a. Multivariate Cox's regression analysis of clinical and karyometric features with survival of patients with a renal cell carcinoma.

	ß	$SE_ß$	*P*	relative hazard
Clinical features				
UICC-stage (1987)	0.875	0.225	0.001	2.398
Karnofsky score	-0.039	0.014	0.006	1.041
weight reduction	-0.740	0.280	0.008	2.096
Karyometric features				
D SDAREA (anisokaryosis)	0.044	0.018	0.018	1.045

Table 4b. Calculation of the RCC-score based on the features found in the Cox's multivariate regression analysis and number of the patients in the prognostic groups based on the RCC-score.

	XBeta =	(0.875 * stage) +
		(-0.040 * Karnofsky score) +
		(-0.740 * weight reduction) +
		(0.044 * D SDAREA)

stage	: UICC-stage (1987)
Karnosfky score	: performance status (0-100)
weight reduction	: weight reduction prior to diagnosis (=1), else 2.
D SDAREA	: difference of SD of nuclear size within the tumor.

RCC-score	XBeta	n	3-yrs survival (%)	range
good prognosis	≤ -2.3	39	80	(65-95)
intermediate prognosis	-2.3 - -1.0	54	41	(27-55)
poor prognosis	> -1.0	28	32	(6-37)

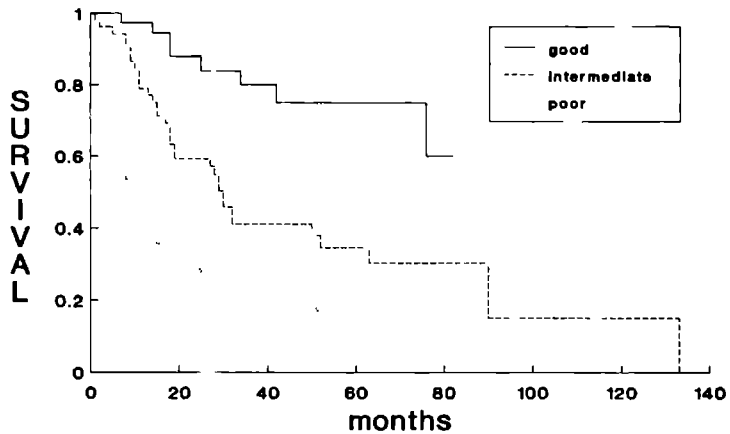

Figure 3. Kaplan-Meier survival curves of different prognostic groups according the RCC-scores (good-prognosis group: n=39, intermediate prognosis: n=54, and poor prognosis: n=28).

DISCUSSION

The main problem in the management of patients with RCC is the unpredictable clinical course of the tumor. Since tumor grading of RCC is hampered by considerable tumor heterogeneity we investigated a karyometric grading system for the quantification of differences within the tumor presumably associated with heterogeneity. The karyometric grading was tested for its additional prognostic value to classical characteristics.

Karyometric analysis, i.e. the quantification of cell nuclear features in light microscopy has been successfully applied for the grading of several cancers. In renal cell carcinoma, Tosi and associates and Bibbo and associates found a positive correlation between morphometric results and survival in patients with stage-I disease.[15,16] Murphy and associates described a nuclear shape analysis which allowed the correct assignment of outcome of localized carcinoma which may become available in clinical practice.[17] From the studies done so far it has become clear that nuclear size as well as nuclear shape are of prognostic value in RCC. However, the number of patients in these studies was rather small. Moreover, tumor areas for analysis were often chosen at random and none of the studies distinguished different parts within the tumor.

From flowcytometric studies[10,18] we learned that considerable heterogeneity of tumor cell populations exists in RCC. The origin of multiple cell populations within the same tumor can most likely be found in genetic instability, a trait of aggressive neoplasms. Therefore tumor grading systems including measures for tumor heterogeneity as the Gleason grading for prostate cancer,[14] may be of additional prognostic value. Karyometric analysis offers a means to quantify nuclear features within the cell clones. Hence, the technique can be used to obtain a measure for the level of phenotypical differences between the cell clones within the same tumor. We postulated that the degree of nuclear phenotypic differences within the tumor as assessed by karyometric analysis is indicative for tumor heterogeneity and thus may in fact correlate with genetic instability in the tumor development. Since increased genetic instability might result in more rapid progression to malignant, e.g. metastasizing phenotype the karyometric grading of tumor heterogeneity might be a good predictor of tumor malignancy.

In the present study morphologically different areas within the tumor were selected by the pathologist and karyometrically analyzed. Whereas the number of

marked tumor areas per tumor was of no prognostic value, data from our study indicate prognostic value for the degree of heterogeneity as assessed by the differences of karyometric phenotype between subpopulations. Heterogeneity in DNA content (2cDI), nuclear size, shape, and chromatin pattern were all correlated with survival, which is in agreement with other studies.[15-17]

To evaluate the additional value of karyometric tumor grading to classical tumor characteristics we also analyzed several clinical characteristics.

The stage of the tumor, including invasion of the vena cava, and the presence of tumor bearing lymph nodes or metastases allows to make subdivisions of patients with regard to survival. The local extent of tumor at the time of surgery is the most important single variable in determining survival.[19] Because the TNM(V) classification defines the anatomic extent, it is often at the basis of further stratification of risk factors.[19-21] The patients in the current study were classified according to the TNMV and Robson system and it appeared that a higher stage was related to a lower survival. More explicitly, not only tumor stage but also the presence of lymph node or distant metastases showed to be an important prognostic indicator for survival. In the univariate analysis they all appeared to be of prognostic significance. It is now clear that vena cava invasion, when curatively operated, is not a prognostic indicator as such.[22] The reason is that it is often associated with factors that heavily influence survival, as was also seen in our study.[20]

Other features, related to survival, were performance status (Karnofsky score) and a history of weight reduction. These obvious clinical characteristics were also found by others, and should be included in a prognostic factor analysis.[19,21] Size of the primary lesion, indirectly related to the T-stage, was not an independent prognostic factor, as was also seen by others.[21] RCC are divided in the literature into clear cell, granular cell, spindle cell, and oncocytoma type tumors. Although oncocytomas are characterized by a favorable clinical course, the prognostic importance of the other tumor types is not clear.[22-27] In our series oncocytomas were excluded and no difference in correlation with survival was found for the other tumor types.

To obtain features that independently predict survival, a multivariate analysis was performed. It was obvious that tumor stage, history of weight reduction, and the karyometric features that illustrate the heterogeneity of the tumor appeared to be the most important prognostic factors in patients with RCC. With these factors we were able to define certain risk groups that can be used for treatment decision and the development of future clinical trials.

In tumors already metastasized at time of diagnosis (stage IV) karyometric analysis appeared to be the best predictor of survival. However, the karyometric features describing heterogeneity in the tumor were less predictive of survival in this patient group. It is therefore tempting to speculate that in metastasized disease malignant behaviour of the tumor is more determined by the presence of malignant cell clones rather than by the chance of development of an even more malignant phenotype as possibly represented by tumor heterogeneity. In this light, we consider high-stage tumor as completely dedifferentiated cancer that gained full malignant potential.

We conclude that clinical and karyometric features correlate with tumor behaviour. Moreover, a combination of karyometry and clinical data offer the best prediction of survival. Tumor heterogeneity, as quantified by karyometric analysis plays an important role in tumor behaviour. The factors analyzed in this study may be of use to individualize the treatment of patients with RCC.

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Chapter 6

TUMOR HETEROGENEITY AS PROGNOSTIC FACTOR IN PATIENTS WITH LOW-STAGE ($T_{1-3}N_0M_0$) RENAL-CELL CARCINOMA

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ABSTRACT

Of patients presenting with a renal cell carcinoma (RCC) the tumor is localized ($T_{1-3}N_0M_0$) to the kidney in 50% of cases. The risk of progression after radical tumor nephrectomy in these patients is 45-65%. In order to predict the chance on recurrence or development of metastases after nephrectomy tumor markers are necessary. Besides tumor stage, tumor grade was shown to be of prognostic value. Tumor heterogeneity, however, may render visual grading systems difficult and subjective. In the present study we investigated a quantitative grading system focusing on objective measures for tumor heterogeneity. The karyometric grading system was correlated to follow up data and tested for its additional predictive value to clinical and pathological tumor characteristics.

Material from 52 patients with $T_{1-3}N_0M_0$ tumors was studied. During follow up of at least 3 years, 21 patients developed local recurrence or distant metastases. The tested clinical and pathological features were age, sex, weight loss, performance status, serum haemoglobin concentration, tumor stage, tumor size, and histological tumor type. Multiple areas per tumor were karyometrically analyzed on cell nuclear features to obtain a measure of heterogeneity of nuclear features within the tumor.

In an univariate Cox analysis for risk of progression, of the clinical and pathological characteristics only performance status appeared to have prognostic significance; the karyometric characteristics of nuclear shape, nuclear size, and chromatin patterns were also predictors of tumor progression. In a multivariate analysis differences in chromatin pattern within the tumor were the best predictors of progression; only T stage added to the prognostic significance.

In conclusion, heterogeneity of chromatin patterns within the tumor as assessed by karyometric analysis appeared to be the tumor feature strongest correlated with tumor progression in patients with a localized RCC. Only tumor stage offered additional prognostic value in this model.

INTRODUCTION

In approximately 50% of patients with renal cell carcinoma (RCC) the tumor is confined to the kidney or the surrounding tissue.[1] Notwithstanding the sometimes large size of the tumor at time of diagnosis, complete macroscopical removal of the tumor can often be achieved by radical surgery. The chance of local recurrence or development of distant metastases, however, is still 45-65%.[2,3] Moreover, the prognosis of patients with progressive disease after radical tumor nephrectomy for a locally confined tumor is very poor: mortality rate of 74% at 1 year.[3] Hence, the overall 5-year survival rate for localized renal cancer ranges from 50 to 90 %.[1,4-6]

Earlier studies revealed several predictors of tumor progression in renal cell carcinoma.[7-9] Tumor stage is considered to be the most important factor.[9] However, in localized tumors, stage alone could not predict outcome.[1] Histological tumor features like tissue architecture, cell type, and tumor grade may be of prognostic value.[6,8,10,11,12] Papillary growing tumors have been reported to have a more favorable prognosis,[11] but pure papillary tumors are rare,[13] which may be the reason for contradictory reports.[7,14] Of the different cell types spindle-cell tumors exhibited a poor prognosis compared to granular and clear cell carcinomas, the most predominant cell types found.[8,13,15] The latter two cell types are often found concomitantly in the same tumor rendering tumor typing more difficult. Besides the distinction of oncocytomas and spindle-cell tumors the subdivision of RCC on base of histological and cytological characteristics is of little prognostic value.

However, tumor grading of RCC proved to be useful.[7,16] In particular nuclear atypia was correlated with tumor behavior[7] and quantitation of nuclear shape by image analysis techniques (karyometry) had predictive value in patients with localized disease.[2,17]

Heterogeneity of tumor cell populations within a tumor, as is indicated by the high percentage of mixed-cell tumors and shown in flow-cytometric analysis,[18] will affect tumor grading. The origin of different phenotypes within the tumor is not clear and the influence of this phenomenon on tumor prognosis has not been studied extensively.[13,19] Due to heterogeneity, grading of only one part of the tumor, as is normally performed in RCC, might not be sufficient, whereas grading of multiple tumor areas is time consuming, tedious, and may have detrimental effect on the already low reproducibility of tumor grading systems in general.[20,21] Quantitative analysis of nuclear DNA content by flow cytometry seemed rewarding in assessing

both tumor heterogeneity and prediction of behavior[18,22,23] although results are equivocal.[24] Image analysis in light microscopy enables analysis of a wide range of nuclear (karyometry) and cellular features (cytometry), including nuclear DNA content.[25] Since these can be assessed in different parts of the tumor image analysis can be used for quantitation of tumor heterogeneity.

In many clinical trials the role of immunotherapy for disseminated RCC has been studied.[26] The low response rates found (14-30%) may be caused by positive response in only a selected group of patients.[27,28] It appeared that especially patients with: good performance status, relatively low tumor burden,[29] no central nervous system or bone metastases, and a long interval between nephrectomy and appearance of metastases benefit from immunotherapy.[30,31] These characteristics often apply to patients with localized disease, who therefore might be good candidates for early immunotherapy.[32] Considering the toxicity of these therapy regiments, however, careful selection of patients on base of prognostic factors is mandatory.

Since tumor progression after radical surgery will probably be due to outgrowth of micro-metastases that already exist at the time of tumor nephrectomy but can not be detected with present diagnostic tools, other predictors of tumor progression are needed. To find prognostic factors we studied clinical, pathological, and karyometric features for correlation with tumor progression in patients with localized renal cancer. Karyometric analysis was applied to obtain quantitative information on tumor heterogeneity.

MATERIAL AND METHODS

Patients

We analyzed data of 52 patients (31 men; 21 women) with clinically localized RCC treated between 1983 and 1989 with radical nephrectomy. The median age was 57.4 years (range, 29-93 years), followup was at least 3 years (median, 60 months).

No evidence of lymph node metastases was found in pathologic examination of lymphadenectomy specimens and no distant metastases could be detected at routine examination including computed tomography of abdomen and lung tomography. Tumors were classified according to the tumor-nodes-metastases (TNM) system developed by the International Union against Cancer, which classifies cancer cases according to the anatomic extent of disease (Hermanek et al., 1987). Localized disease was defined as stage $T_{1-3}N_0M_0$. As an endpoint in follow up tumor progression

was studied. Progressive disease was defined as renal fossa recurrence or development of distant metastases.

Clinical features

From literature study several clinical features were chosen for correlation with follow up data. The features included were patients age, performance status (Karnofsky score), a history of weight loss, and serum haemoglobin concentration at time of diagnosis. Besides clinical features the following pathologic characteristics were recorded: T stage, tumor size, and histologic tumor type.

Histological material

Depending on tumor size 10 to 20 blocks per tumor were cut from all tumor parts. Material of the resected tumor parts was formalin-fixed (buffered, 4%) and embedded in paraffin. Two consecutive 4 μm slides were cut from each block of the resected tumor. One slides was used for hematoxyline staining and interpretation by the pathologist. The second slide was stained according to Feulgen (5 N HCl, 60 min. and Schiff's reagent, Merck, 30 min. both at room temperature) and used for karyometric analysis. The slides were cover-slipped (mounting medium: Permount SP15, Fisher Scientific).

Karyometric features

One pathologist (H.E.S.) reviewed all histological material and marked the morphologically different tumor areas for karyometric analysis. The selection of areas was based on differences in histology (papillary and non-papillary), cytology (clear, granular, and spindle cell), and degree of nuclear atypia. Since this is an important step in the quantitative grading that introduces a subjective element we tested the selection of tumor areas inter- and intraindividually by two pathologists in 10 randomly selected tumor samples. Karyometric analysis consisted of nuclear morphometry, densitometry, and chromatin pattern analysis (see Table 2 and 3 of Chapter 1) with a PC-based image-analysis system (VFG-framegrabber board, Imaging Technology, Woburn, MA) in a Compaq 386s personal computer. Image handling and analysis software was written in TIM (TEA, Dordrecht). Images of 512x512 pixels were digitized and stored on computer hard-disk prior to analysis. In the image analysis steps the images were corrected for background shading and filtered for segmentation applying local segmentation routines based on grey value

histogram interpretation (iso-data threshold determination). After thresholding, several binary operations were conducted including detection and separation of overlapping objects. Of each nucleus a panel of morphometric, densitometric, and chromatin textual features calculated (Table 2 and 3 Chapter 1) and the automaticly contoured and numbered nuclei were marked in the image for verification by the technician. Of each tumor area 50 images including 50 to 200 nuclei were analyzed. Lymphocytes in the slide were used as reference for 2c DNA content after applying a correction factor of 1.19.

To assess the degree of nuclear tumor heterogeneity we applied the following mathematical interpretation of the karyometric analyses of the different areas per tumor. In all tumors for every nuclear feature the karyometric feature value of the tumor area with the highest and lowest value and their differences was determined resulting in karyometric measures of nuclear differences within the tumor. To study the effect of separate analysis of tumor areas compared to analysis of more random measurements, all data per tumor were merged and median and difference of 15th and 85th percentiles were calculated to obtain a more subpopulation independent measure of tumor heterogeneity.

Statistical methods

For analysis of reproducibility the ANOVA test was applied. As an endpoint in follow up tumor progression was studied. The Kaplan-Meier method was used to estimate the progression rate. Differences obtained were tested for statistical significance with the log-rank test and the Wilcoxon test. The 3-year tumor progression rates, with 95% confidence intervals, were calculated.

Cox's univariate and multivariate regression analyses (i.e., proportional hazard model) were performed to estimate the influence of the clinical and karyometric features on time to tumor progression. Both forward and backward stepwise selection procedures were used to find the best model for predicting the disease-free interval. The selection procedure was performed in two parts. In part I, the selection was used to find all clinical variables to define either the best or an equivalent model. The Bonferroni correction was used for model entry of a variable. The same procedure was also performed on the karyometric feature groups. In part II we used the selection procedure on both clinical and karyometric features selected in the previous step.

RESULTS

Clinical features

Progressive disease occurred during follow up in 21 (40%) patients, of which 15 (71%) died of disease. The 3-year progression rate was 33% for T_1 (n=5), 29% for T_2 (n=23), and 47% for T_3 (n=24). The differences between the progression rates for the tumor grades were not significant in the Wilcoxon test ($P = 0.19$) or the log-rank test ($P = 0.17$).

Tumor stage, patient age and sex, serum haemoglobin concentration at the time of diagnosis, histologic tumor type, tumor size, and history of weight loss, were not significantly related to progression ($P>0.10$) in the univariate Cox regression analysis. Of the clinical features only Karnofsky score was significantly correlated with progression ($P < 0.05$), i.e. patients with a low Karnofsky score at the time of diagnosis were at risk to develop tumor progression (Table 1).

Table 1. Univariate Cox's regression analysis of clinical features and tumor progression.

	Beta	SBeta	P	R
Sex	0.674	0.450	0.1343	0.041
Age	-0.008	0.016	0.6241	0.000
Karnofsky score	-0.051	0.243	0.0369	-0.137
Weight loss	-0.772	0.487	0.1130	-0.064
Haemoglobin	-0.100	0.178	0.5722	0.000
T stage	0.686	0.408	0.0927	0.076
Tumor size	0.094	0.071	0.1836	0.000
Tumor type	0.350	0.520	0.5006	0.000

Karyometry features

In the univariate Cox analysis no relation was found between the absolute number of marked tumor areas and progression. Of the nine karyometric features, that significantly correlated with tumor progression eight were features calculated from the different tumor areas and one (MEDPASS, median of nuclear shape descriptor PASS of all tumor areas in the tumor) was a feature weighted for the number of nuclei analyzed in the different areas (Table 2). Differences in karyometric-feature values between different tumor areas within the tumor were highly correlated with progression, illustrating the prognostic value of karyometricly assessed tumor

heterogeneity.

Table 2. Karyometric features significantly correlated with tumor progression in univariate Cox regression analysis.

	Beta	SBeta	P	R
L[@]2cDI[$]	-0.234	0.117	0.0464	-0.118
U MBEN	2.243	1.108	0.0429	0.122
D SDAREA	0.102	0.041	0.014	0.170
D MSCAT	1.281	0.454	0.0048	0.206
D MH1	5.934	2.009	0.0032	0.219
D SDH1	6.322	2.424	0.0091	0.185
D MH3	60.002	19.386	0.0020	0.232
D MH5	2.799	1.001	0.0051	0.204
MEDPASS	-0.254	0.121	0.0351	-0.132

Note[@]): L= lowest population value
U= highest population value
D= U-L
MED= median value for entire tumor (all populations merged)

[$]) 2cDI = 2c Deviation Index (Böcking,1984)
MBEN = mean bending nuclear shape factor (based on smoothed differences in Freeman chain code), describing nuclear irregularity.
SDAREA = standard deviation of nuclear area
MSCAT = mean scatter: variance in pixel values (texture feature)
MH1 = mean H1 (Markovian feature: entropy)
SDH1 = standard deviation H1
MH3 = mean H3 (Markovian feature: inverse difference moment)
MH5 = mean H5 (Markovian feature: inverse rotation moment)
MEDPASS = median value in tumor of PASS (nuclear shape factor based on smoothed differences in Freeman chain code)

Reproducibility analysis showed discrepancies for only three of 32 karyometric features among three technicians: the same observer scored significantly higher values for one feature (MH3) and differences were found for two other features (MAREA, MIOD). This resulted in a higher percentage of explained variance due to the observers compared to the total variance (2%, 6%, 8% respectively). For the other features the explained variance was less than 1%. Comparison with the percentage explained by the variation between slides showed that differences between observers might be of minor importance: percentages explained by the variation between slides

was 85%, 71%, and 68% respectively for the features (MH3, MAREA, MIOD) and 78% to 93% for the remaining features.

Multivariate analysis

Clinical, pathological and karyometric features were finally tested in a multivariate analysis. A selection of features had to be made in order to reduce the number of variables in the analysis. Selection was done on base of results from the univariate analysis and findings in earlier studies. The selected features were: presence of weight reduction, patients sex, Karnofsky score, tumor stage, and the karyometric features: 2c deviation index (2cDI), highest mean of BEN and PASS (nuclear shape descriptors) in the tumor, and the differences within the tumor between the analyzed areas of the following features: standard deviation of nuclear profile area, mean SCAT value per area, mean and standard deviation of entropy (Markovian feature 1.), mean of inverse difference moment (Markovian feature 3.) and rotation moment (Markovian feature 4.).

The difference between tumor areas within the tumor of the chromatin pattern descriptor, mean of the inverse difference moment of the co-occurrence matrix (Markovian feature 3.) was the best predictor of tumor progression in this model (Table 3). Although substitution of one of all other karyometric features, except the 2cDI in the model resulted in only slightly inferior predictive value. Otherwise, only T stage showed additional prognostic significance. In the multivariate analysis, sex, age, presence of weight loss at the time of diagnosis, histologic tumor type, tumor size, and Karnofsky score did not add to prognostic significance.

Table 3. Multivariate Cox regression analysis of clinical and karyometric features and tumor progression.

Step	Beta	SBeta	P
1. D MH3*	61.330	19.90	0.002
2. Tumor stage	1.086	0.492	0.027

* Differences between populations with highest and lowest values of the inverse difference moment of the co-occurrence matrix (Markovian texture feature) within tumor.

DISCUSSION

The presence of multiple morphologically different tumor areas in renal cell carcinoma is a known phenomenon. Histological as well as cytological patterns can differ widely within a single tumor. In a population of 162 cases Delahunt et al. (1987)[13] found only 23% with a solely papillary growth pattern. Medeiros et al. (1988)[8] found large intra-tumor variation in cytological patterns whereas Fuhrman et al. (1982)[7] revealed differences in the level of nuclear atypia. Tumor grading systems for RCC often only consider the least differentiated part of the tumor regardless of the presence of other tumor parts. In order to obtain a measure for heterogeneity comparison of tumor areas is necessary. Visual grading based on cellular and nuclear features does not enable quantitative comparison. Since nuclear atypia was correlated to cytological cell type[13] and nuclear grading proved a powerful tool for grading RCC[7] we choose quantitative analysis of nuclear phenotype to assess tumor heterogeneity. In this approach morphologically different tumor areas are compared. We should bear in mind, however, that different cellpopulations in heterogeneous tumors do not necessarily grow in separate tumor parts and mixing of populations may occur. Therefore we also considered the variation of nuclear phenotype within each tumor part.

Karyometric analysis has been shown to be of prognostic significance. Several authors found a positive correlation between morphometric results and survival in patients with stage-I disease.[2,17,34] Others found a prognostic discriminant in the nuclear size of all RCCs.[35] Murphy and associates (1990)[2] described a nuclear-shape analysis that predicts outcome of a localized carcinoma and might become available for clinical use.

In the present study we found several karyometric features to correlate with tumor progression in localized tumors. Again nuclear shape was significantly correlated with tumor behaviour. When compared with measures of tumor heterogeneity, however, these were the strongest predictors of tumor progression. The presence of heterogeneity in nuclear size (anisokaryosis) in the different tumor areas was significantly related to tumor progression. The importance of heterogeneity is also illustrated by the results of the chromatin-texture analyses: recurrence or progression is earlier in patients with a high variance in nuclear chromatin patterns as measured by the Markovian texture features in the different tumor areas.

To obtain features that predict recurrent disease independently, we performed a

multivariate analysis. Of the clinical and karyometric characteristics, the features describing variance in chromatin pattern within a tumor were significantly correlated with the chance of local recurrence or of development of metastatic disease in the multivariate model. Of all the other clinical features, only tumor stage yielded additional information.

Although tumor heterogeneity has been described for several tumors and its correlation with prognosis has been reported, we can only speculate on the reason for the worse prognosis of heterogeneous tumors.[36,37] A cause of tumor heterogeneity can presumably be found in genetic instability in the tumor resulting in development of several viable tumor cell populations with increased risk on development of a cell population with a more malignant phenotype.

The presence of large differences in phenotype between populations, as can routinely be described by karyometric analysis, could be a useful predictive tool. The karyometric features were tested as prognostic factors in a patients population with localized RCC (T_{1-3} N_0 M_0). The chance of local recurrence or development of distant metastases in patients with locally confined RCC is 45-65%.[2,3] Moreover, the prognosis of patients with progressive disease after radical tumor nephrectomy for a locally confined tumor is very poor: a mortality rate of 74% at 1 year has been recorded.[3]

Many clinical trials have studied the role of immunotherapy in patients with RCC.[26] In patients with metastatic disease, 14-30 % reacted positively to this type of therapy.[27,28] Especially patients with good performance status, relatively low tumor burden, no central nervous system or bone metastases, and a long interval between nephrectomy and appearance of metastases seemed to benefit from immunotherapy.[30,31] Because patients with a low tumor burden react better to immunotherapy, patients with localized disease might benefit from this kind of treatment. The karyometric analysis of routinely processed slides can offer additional information and may be used to identify patients at risk. In a prospective randomized trial testing adjuvant immunotherapy in localized RCC the method will be further analyzed as a prognostic tool.

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Chapter 7

KARYOMETRIC ANALYSIS OF INTRA-TUMOR HETEROGENEITY IN PROSTATE ADENOCARCINOMA

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ABSTRACT

The intra-tumor heterogeneity in different locations in the prostate was determined by karyometric image analysis and compared with local tumor progression in a retrospective analysis of 65 patients with localized adenocarcinoma of the prostate. In these 65 radical prostatectomy specimens 290 tumor locations were documented. Of each location tumor volume was estimated and Gleason grade determined. Quantitative image analysis of nuclear size, shape and chromatin pattern was performed. As measures for intra-tumor heterogeneity, differences in Gleason grade and karyometric feature values were evaluated. Moreover, of each tumor area the location within the prostate was documented. Gleason grade and karyometric features varied widely in the prostate. Tumor locations in the apex (n=92) had significantly larger and more irregular-shaped nuclei ($P < 0.005$) compared with basally located tumors (n=49). Significant differences in nuclear shape were also found in different locations in the equatorial plane of the organ. Tumor heterogeneity in chromatin pattern features was found to correlate with local extension (seminal vesical invasion, extracapsular tumor growth, positive resection margins) and lymph node metastases. This correlation was even stronger in case more pathological features were present. In view of the observed tumor heterogeneity, (karyometric) analysis of multiple tumor areas in the prostate is advisable for optimal evaluation of prostate carcinoma.

INTRODUCTION

Prostate cancer is the second cause of cancer-related death in males. However, only 1 of 3 patients with prostate cancer will finally die of the disease.[1] The increase in life expectancy and the improvement in diagnostic facilities are the main factors responsible for an increase in the number of patients diagnosed with prostate cancer.

More than 50% of the patients have metastatic disease, at the time of diagnosis.[2] Curative treatment modalities for these patients are lacking and prognosis is poor.[3] At the other end of the malignancy spectrum we encounter tumors confined to the prostate. These low-stage cancers can be divided into: latent carcinoma, found at postmortem examination; incidental carcinoma, found after resection for benign prostatic hyperplasia; and clinical prostate cancer, diagnosed by either digital rectal examination, PSA serum test, or ultrasound imaging.[4]

It has been postulated that the different types of localized cancer are related to their anatomical location within the prostate.[5] The prostate is composed of three zones: the peripheral, central, and transition zone. Tumors originating in the peripheral zone would be more readily diagnosed by digital rectal examination, whereas, incidental cancer found in transurethral resection material develop in the transition zone of the prostate. Recently, Greene et al. (1991)[6] observed aneuploidy at lower cancer volume in peripheral zone cancers compared to transition zone tumors. These findings indicate that both the anatomical location and biological differences within the prostate may play a role in the malignant potential of localized cancer lesions in the prostate.

The location of the tumor within the prostate also is of influence on the pattern of capsular penetration. In the basal part of the prostate the neurovascular bundle runs distally through the capsula before entering the prostate, whereas in the apical part short branches innervate directly through the capsula. Hence, neural invasion in the basal area is only rarely accompanied with positive resection margins, whereas, apical invasion of the neural branches more rapidly results in extensive extracapsular tumor growth.[7]

Which factors may predict the malignant behaviour and development of progressive disease in localized prostate cancer after radical prostatectomy? Five-year recurrence rates after prostatectomy increase with tumor stage ranging from 0% for small incidental cancers (stage A1 or T1) to 8% for stage B2 or T2c cancer.[8] Seminal vesical involvement and capsular penetration negatively influence prognosis.[8-10] Tumor

size was an important predictor of metastases; no metastases were found in tumors smaller than 4 cc.[11] In addition to tumor size, grade provides prognostic information.[7] Unfortunately grading of prostate cancer was found to be of low reproducibility.[12] Quantitative interpretation of cell nuclear features by image analysis techniques (karyometry) showed correlation of nuclear size and shape with prognosis and improved grading reproducibility.[13,14] In particular variation of karyometric features within the tumor were of predictive value.[14] For renal cell carcinoma we applied image analysis techniques for quantitative assessment of phenotypic nuclear heterogeneity, a possible result of genetic instability. Karyometric features describing heterogeneity were important predictors of tumor progression.[15]

In the present analysis we studied karyometric features in prostate tumors after radical prostatectomy to answer the following questions: 1. Are karyometric tumor characteristics related to the location of the tumor in the prostate; 2. What is the degree of karyometric heterogeneity in localized prostate cancers; 3. How do karyometric features correlate with clinical tumor characteristics.

MATERIAL AND METHODS

A retrospective analysis of 65 radical prostatectomy patients with a median follow up of 3 years (range 2-11 years) was performed. Clinical and pathological data were recorded. For staging the TNM classification was applied[16] (Table 1). For clinical staging, digital rectal examination, serum PSA level, and transrectal ultrasound were used. Each patient was routinely screened using bone scan and CT scan for the presence of metastases.

The prostatectomy material was routinely processed (formalin fixation and paraffin embedding). A mean of 11.4 blocks (range 5-23) per prostatectomy specimen was available. The prostatectomy material was cut as follows: the apical and basal cap were laminated in ventral-dorsal direction; the lobes of the organ were laminated in the equatorial plane. Although archival material was used and whole organ mounts were not available, determination of location of the tumor was almost always possible, since location of each block was documented in the pathology report. In case the location of a block was hazardous (n=8) the block was used in the analysis of tumor heterogeneity, but excluded for comparison of location. For each case the presence of seminal vesical invasion was documented as well as tumor growth outside the prostate

Table 1. The TNM-classification system for prostate adenocarcinoma.[16]

Tumor extent	T	Lymph nodes	N
- incidental tumor:			
+ < 5% of resected tissue	1a	- can not be assessed	x
+ > 5% of resected tissue	1b	- no lymph nodes	0
		- one lymph node (< 2cm)	1
- tumor identified by needle biopsy	1c	- (multiple) node(s) (2-5cm)	2
		- nodes > 5cm	3
- tumor confined within prostate			
+ half a lobe or less	2a		
+ more than half a lobe	2b		
+ both lobes	2c		
		Metastases	M
- tumor extends through capsula			
+ unilateral	3a		
+ bilateral	3b	- can not be assessed	x
+ invades in seminal vesical	3c	- no distant metastases	0
		- distant metastases	
- tumor fixed to adjacent structures		non-reg. lymph nodes	1a
+ bladder neck external sphincter		bone	1b
rectum	4a	other site	1c
+ levator muscles pelvic wall	4b		

capsula. A mean of 8.5 lymph nodes (range 2-21) per prostatectomy was removed and histologically examined for metastases. All hematoxylin-stained slides per patient were reviewed and each tumor area was marked on the slide and the location in the tumor was documented. For location purposes the following division of the prostate was made: 1. left or right lobe; 2. apical (caudal 1 cm of prostate), central, or basal part (cranial 1 cm of prostate); 3. dorsal, ventral, or lateral; 4. peripheral, transition, or central zone. The size of the tumor location was expressed as percent of each block and estimation of tumor volume in cc was made using a grid placed over the microscopic slide. In the 65 prostatectomy specimens, 290 tumor locations were documented (mean 4.5, range 1-15).

Karyometric analysis

For karyometric analysis 4 μm slides were cut adjacent to the hematoxylin stained material. Sections were mounted on slides and Feulgen staining was applied: 5 N HCl, 60 min. and Schiff's reagent 30 min. both at room temperature. The marked tumor

areas were copied on the Feulgen-stained slides. The image analysis system consisted of a routine light microscope (Axioskop, Zeiss, Oberkochen, Germany) mounted with a CCD-videocamera (HCS, Eindhoven, The Netherlands) connected to a framegrabber board (VFGplus-AT, Imaging Technology, Woburn, MA, USA) in a personal computer (Compaq 386s, 16 MHz, Houston, Tx, USA). The karyometric analysis consisted of recording of 50 images per tumor area using manual focussing using a 40x objective (NA 0.75). Automaticly the nuclei present in these images were segmented and analyzed by the system for 32 nuclear features including nuclear size and shape features (morphometry), DNA content analysis (densitometry), and analysis of chromatin pattern (texture analysis, Markovian features) (see Table 2 and 3, Chapter 1). The original image (512 x 512 pixels, 8 bits) was corrected for shading by subtracting a background image. For smoothing of boundaries an uniform and percentile filter were applied on the black-and-white digital image. Segmentation of nuclei was performed applying local thresholding procedures (isodata thresholding) for optimal correction of local background illumination. After the analysis, the segmented nuclei were contoured and numbered in the original images enabling visual verification. In this way measurement of artifacts and out-focus nuclei could be avoided. The reproducibility of this system was tested earlier and found acceptable.[15]

DNA histograms were analyzed using the 2c Deviation Index (2cDI) and the 5c Exceeding Rate (5cER) as defined by Böcking.[17] Each histogram was scored as Aneuploid, when the main peak was over 2.5c DNA content, or Diploid, when the main peak was in range of 1.5 to 2.5c.[18]

Prognostic groups

Since the clinical follow up of most cases was too short to allow analysis of progression, karyometric features were compared to four known pathological features: seminal vesical invasion, extracapsular tumor growth, positive surgical margins, and lymph node metastases.[8] Moreover, the patients were grouped as follows: group A: none of the above pathologic features present (n=26); group B: one pathologic feature present (n=16); group C: two pathologic features (n=14); group D: three pathologic features (n=6); group E: all features present (n=3).

Statistical analysis

Of all karyometric features mean and coefficient of variation were calculated for the analyzed tumor areas. The mean, minimal, and maximal value for the analyzed tumor

areas per patient were calculated to obtain measures for intra-tumor heterogeneity of nuclear phenotype. In each patient the highest and lowest Gleason grade were recorded as well as the distribution in tumor volume of the different tumor areas. For comparison of different locations the Student's *t*-test and the ANOVA test were applied. The Mann-Whitney *U*-test was applied to compare karyometric feature values in the different prognostic groups. Comparison of multiple features was done in logistic regression analysis using the Statistical Package for the Social Sciences software (SPSS/PC+ version 4.0, Chicago, USA). In multiple regression analyses logarithmic values of the karyometric features were calculated.

RESULTS

Tumor stage and grade

The distribution of clinical and pathological stage is given in Table 3. Correct clinical staging in T_2 and T_3 tumors (n=53) was obtained in 32 cases (60%), 3 cases (6%) were overstaged, and 18 cases (34%) were clinically understaged compared to the pathological stage (Table 3). The correlation of grading of the initial needle biopsy, when available, and the prostatectomy specimen is given in Table 4. Overall agreement in differentiation was found in 26 cases (49%). The biopsy material resulted in undergrading in 21 cases (40%) and in overgrading in 6 cases (11%) compared to prostatectomy specimen.

Table 2. Comparison of clinical T-stage and pathological stage in 65 radical prostatectomy specimens. Prostatectomies after diagnosis of tumor in TURP material were clinical stage-1 tumors. The actual pathological stage of the prostatectomy specimens in these cases (n=9) is presented. Three carcinoma were found in prostate material of cystectomy specimen and clinically staged T_e.

	Pathological stage							
	2a	2b	2c	3a	3b	3c	4a	total
clinical stage								
T0	1		1	1				3
T1	3		3	1	1	1		9
T2	7	2	12	6	3	8		38
T3	3			6		5	1	15
	14	2	16	14	4	14	1	n=65

Seminal vesical invasion was found in 17 cases (26%), extracapsular tumor growth in 28 (43%), positive lymph nodes were found histologically in 8 cases (12%), and positive resection margins in 21 cases (32%). Of all tumors, 39 (60%) had at least one aneuploid tumor area (Table 5).

Table 4. Comparison of differentiation of biopsy and the prostatectomy specimen. Biopsy material was available in 53 cases.

	Prostatectomy WD	MD	PD	total
Biopsy*				
WD@	9 (36%)	15 (60%)	1 (4%)	25
MD	4 (17%)	15 (63%)	5 (20%)	24
PD	2 (50%)	2 (50%)		4
				n=53

*) Overall agreement in differentiation was found in 26 cases (49%). The biopsy material undergraded the tumor in 21 cases (40%) and overgraded in 6 cases (11%) compared to prostatectomy specimen.

@) WD = well differentiated; MD = moderately differentiated; PD = poorly differentiated

Prognostic groups

The distribution of the pathological features in the five prognostic groups is presented in Table 6a. Note that nine of 16 cases with only one prognostic feature present had extracapsular tumor growth. On the other hand, most patients with lymph node metastases (5 of 8) were scored in prognostic group D and E, indicating that lymph node metastases occurred mainly in patients that already had other pathological features present (Table 6a). In Table 6b the prognostic groups are compared to the pathological tumor stage. Note the heterogeneity of group B (presence of one pathological feature) as compared to the overall tumor stage.

Table 5. Ploidy pattern in 290 tumor areas in 65 radical prostatectomy specimen.

	Gleason grade 2	3	4	5	n
Diploid	5	106	66	21	198
Aneuploid	2	30	31	29	92

Karyometric analysis and tumor differentiation.

Gleason grade and karyometric analyses were available for 290 tumor areas. A tendency existed for increase in Gleason grade with estimated tumor volume: Gleason grade 2 occurred only in tumors smaller than 1.2 cc (n=7). The location of the tumor was not correlated with differentiation (Table 7). In the univariate analysis, the 2cDI of the separate tumor locations increased with an increase in Gleason grade per location (Figure 1) probably due to a significantly higher percentage of aneuploid tumors in the higher Gleason grades (Table 5).

Table 6a. Distribution of cases in the five prognostic groups (n=65).

	Sem. ves. invasion	Extra Caps. growth	Pos. lymph nodes	Pos. resection margins
Prognostic group				
A (n=26)	0	0	0	0
B (n=16)	2	9	1	4
C (n=14)	6	10	2	10
D (n=6)	6	6	2	4
E (n=3)	3	3	3	3
	n=17	n=28	n=8	n=21

Table 6b. Comparison of prognostic group with overall pathological tumor stage.

	Pathological stage							
	2a	2b	2c	3a	3b	3c	4a	total
Prognostic group								
A	13	1	11	1				26
B	1	1	5	5	2	2		16
C				6	2	6		14
D				2		4		6
E						2	1	3
								n=65

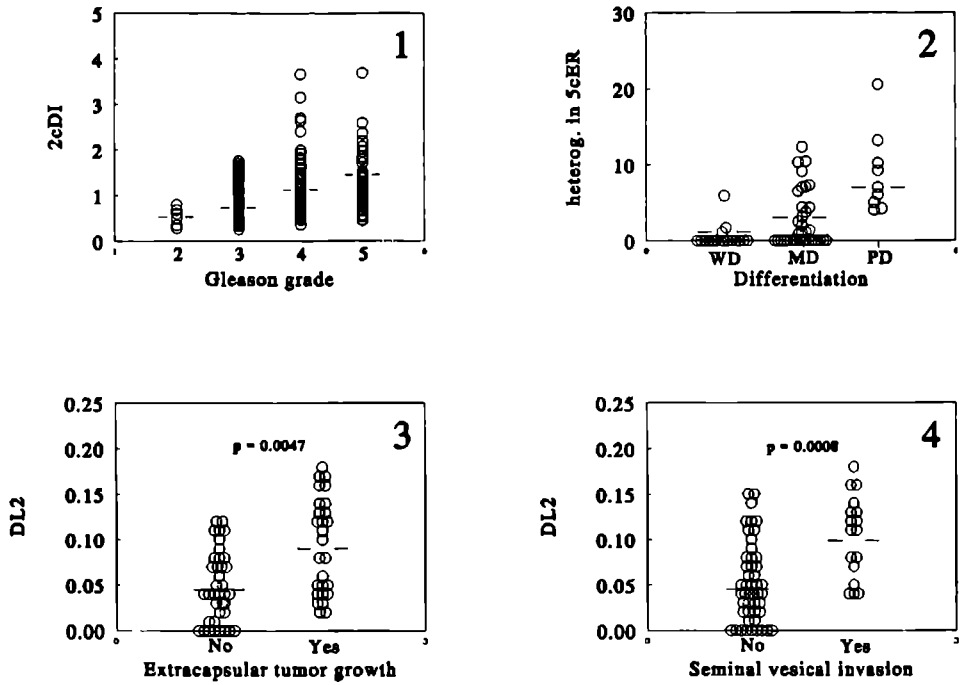

Figure 1-4.

1. Correlation of 2cDI with Gleason grade in different tumor locations (n=290);
2. Correlation of overall tumor differentiation in the 65 prostatectomy specimens with variation in 5cER between different tumor locations within the tumor;
3. Tumors with and without extracapsular tumor growth against heterogeneity in chromatin pattern (Markovian feature: DL2);
4. Tumors with and without seminal vesical invasion against heterogeneity in chromatin pattern (Markovian feature: DL2).

Overall tumor differentiation in the 65 cases was best predicted by the heterogeneity in the percentage of aneuploid cells, as estimated by the variation in 5cER among the measured tumor areas (Figure 2).

Table 7. Distribution of tumor locations in the prostate by Gleason grade of 290 analyzed tumor areas in 65 patients. Zonal location could be determined in 277 tumor locations (96%).

	Gleason grade 2	3	4	5	
Cranial-Caudal locations					
apical	1	49	30	16	
central	3	62	37	24	
basal	3	25	30	10	
					n=290
Dorsal-Ventral locations					
dorsal	2	55	46	20	
lateral	1	21	13	7	
medial	3	50	25	20	
ventral	1	10	13	3	
					n=290
Zonal locations					
peripheral zone	7	124	78	40	
transitional zone	.	4	7	2	
central zone	.	3	7	5	
					n=277

Karyometric analysis and tumor location

The ANOVA analysis revealed differences in karyometric features in the apical, central, and basal tumor locations ($P < 0.05$). Hence, we compared the karyometric features in the apical and basal tumor locations and found significant differences ($P < 0.05$) for five (MMAXD, MFELL, MPASS, MFPE, MSCAT) (Table 8). The results from the karyometric analysis indicated larger and more irregular-shaped nuclei in the apical tumor locations compared to basal areas (Table 8). Gleason grade and tumor volume for apical and basal tumor locations, however, were not significantly different ($P > 0.10$). Karyometric analysis of non-malignant prostate glands (n=10) adjacent to cancer revealed no significant difference in karyometric analysis of basal and apical areas.

Table 8. Mean and Standard Deviation of karyometric features significantly different in apical (n=92) versus basal (n=49) tumors (Student's *t*-test). Only peripheral zone tumors in both areas were compared (n=141). For locations in the equatorial plane 109 tumor locations were compared in ANOVA analysis and p-values for significantly different features are shown.

	Apical (n=92) mean±SD	Basal (n=49) mean±SD	*p*-value *t*-test
MMAXD[@]	8.53±1.23	7.99±1.11	0.011
MFELL	0.720±0.040	0.734±0.036	0.041
MPASS	16.18±1.70	17.01±1.49	0.005
MFPE	0.7328±0.017	0.738±0.013	0.021
MSCAT	6.20±0.67	6.53±0.73	0.008

	Dorsal (n=65)	Lateral (n=28)	Medial (n=5)	Ventral (n=11)	ANOVA F ratio	*p*
MBEN	1.52±0.14	1.42±0.12	1.48±0.07	1.58±0.19	4.353	0.006
MFPE	0.735±0.016	0.743±0.012	0.739±0.008	0.729±0.014	3.561	0.017
MFELL	0.732±0.036	0.746±0.033	0.739±0.022	0.706±0.036	3.457	0.019
MPASS	16.99±1.66	17.40±1.63	16.59±0.72	16.24±1.23	4.286	0.007

[@]) The M at the beginning of a feature name refers to the mean feature value per tumor location.

When tumor locations in the central part of the prostate (n=124) were compared in the equatorial plane, several differences were observed. Nuclear shape was found to be significantly more irregular in the ventral compared to the dorsal and lateral tumor areas as assessed by karyometry (Table 8).

As far as zonal distribution of prostate cancer was concerned, we found most tumor areas located to the peripheral zone (86%) and no karyometric differences were found among the three different zones ($P > 0.05$, ANOVA test).

Karyometric analysis and local tumor extent

To study the relation of karyometric features with tumor stage we calculated for each case (n=65) the maximal, minimal, and mean value for all karyometric features as well as the Gleason grade per tumor area. Seminal vesical invasion and extracapsular tumor growth were correlated with several pathological and karyometric features. Decreased differentiation was accompanied with an increased risk of seminal vesical invasion and extracapsular tumor growth as was the heterogeneity in tumor grade as

Table 9. Differences between tumors with and without the presence of pathological progression markers (seminal vesical invasion, extracapsular tumor growth, positive resection margins, and lymph node metastases) by differentiation and heterogeneity in Gleason grade within the tumor (n=65).

	Sem. ves. invasion		Extra Caps. tumor growth		Pos. lymph nodes		Pos. resection margins	
	No	Yes	No	Yes	No	Yes	No	Yes
Differentiation								
WD	15	2	14	3	15	2	14	3
MD	29	10	19	20	35	4	27	12
PD	4	5	4	5	7	2	3	6
Heterogeneity in Gleason grade[a]								
no difference	26	6	22	10	31	1	25	7
difference = 1	18	6	11	13	19	5	14	10
difference = 2	4	5	4	5	7	2	5	4

[a]) Difference in Gleason grades between tumor area with highest and lowest Gleason grade.

expressed by the largest difference in Gleason grade found between different tumor areas (Table 9).

The presence of lymph node metastases was not related to the differentiation of the initial tumor (Table 9). It should be noted that only a low number (n=8) of patients with positive lymph nodes was present, since, in general, no prostatectomy is performed in patients with positive cryosections of lymph node material during operation. In the patients where prostatectomy was performed with lymph node metastases this was done either because only a very small amount of tumor cells was present in the lymph node biopsies (n=3) or the lymph node metastases were only detected post-operatively, in the total resection preparation (n=5). Tumor volume was significantly higher in patients with extracapsular tumor growth ($P < 0.05$, Mann-Whitney U-test) and seminal vesical invasion ($P < 0.005$, Mann-Whitney U-test). Although a tendency existed towards higher tumor volume in patients with positive lymph nodes, this was not significant ($P > 0.10$). Several karyometric features were correlated with the presence of extracapsular tumor growth. The heterogeneity in chromatin texture (Markovian texture feature L2) was significantly higher in tumors growing through the prostate capsula ($P = 0.0047$) (Figure 3) and invading the seminal vesiculas ($P = 0.0008$) (Figure 4). In a multivariate regression analysis, this was independent of tumor volume and heterogeneity in Gleason grade. The presence

of positive margins at the bladder resection area was significantly correlated with irregular nuclear shapes (P = 0.021) (Figure 5) and nuclear polymorphism (P = 0.0076) (Figure 6).

Correlating the five prognostic groups with the karyometric analysis and pathological findings in a multiple regression analysis again the heterogeneity in chromatin patterns within the tumor, as described by DL2 was selected as best predictive feature (Figure 7).

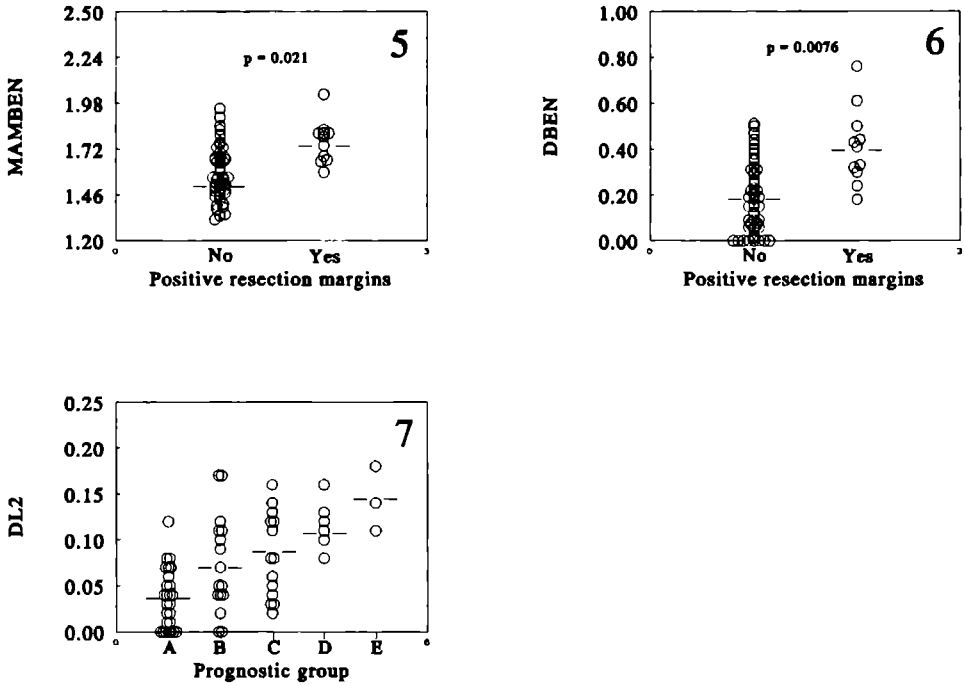

Figure 5-7.

5. Tumors with and without positive resection margins against maximal value of BEN (MAMBEN) (nuclear shape feature higher values represent more irregular shaped nuclei);
6. Tumors with and without positive resection margins against heterogeneity in nuclear shape (BEN);
7. Correlation of heterogeneity in chromatin pattern (DL2) with an increase in number of pathology prognostic markers (prognostic group).

DISCUSSION

The site of origin of the tumor in the prostate may influence tumor phenotype[6] and clinical behaviour.[19] Hence, we studied pathological and karyometric features in different tumor locations within the prostate.

Radical prostatectomy is often applied as treatment for localized prostate cancers.[20] Five year survival for these patients is 75-85% and comparable to an age-matched population.[21] The actuarial rate for the development of distant metastases in localized disease after five years is 7%.[8] These data indicate that local radical therapy although effective for most localized tumors will still be insufficient to control later development of distant metastases in a small number of patients. Besides metastasis, local recurrences threaten the patients' health. Unlike development of metastases, local recurrences can be reduced by radical local treatment.[8] In non-surgically treated patients local progression rates up to 84% at five years have been reported,[22-24] whereas radical surgery reduced this figure to 2-10% in organ-confined and 8-23% in extracapsular disease.[8,9]

The short follow up of patients in our population made progression and survival analysis hazardous.[25] Hence, seminal vesical invasion, tumor growth through the capsula, positive resection margins, and lymph node metastases were chosen as pathological prognostic markers, since these features were previously found to correlate with prognosis.[8,9]

Capsular penetration and seminal vesical involvement in radical prostatectomy specimens were accompanied with an increased risk of local recurrence (8-23%)[8,9] and distant metastasis (30%)[8] at five year follow up, compared to tumors confined to the prostate (0-10% local recurrence and 1% distant metastases).[8,9] Besides local extent of the tumor, grade was of value mainly when used in combination with tumor volume.[7,26] Recent studies indicate that grade alone can be sufficient to predict progression. To circumvent the subjectivity of visual tumor grading, several quantitative techniques have been applied. Flow cytometry,[25,27] tissue PSA levels,[28] and proliferation rates[29] could be used to separate prognostic groups but for the individual patient predictive value was doubtful. The finding of nuclear shape analysis as a prognostic marker is ambiguous. Nuclear roundness factor (NRF) was found by some to be of prognostic value.[13,30,31] But patient populations were small, what may account for the fact that in larger populations no predictive value of NRF was found.[9,14] In combination with DNA content and Gleason grade nuclear shape was not useful to predict local tumor

extent.[32]

When analyzing tumor material, heterogeneity within the tumor should be taken into account[10,15] and may have prognostic value.[10,33] The frequent discrepancy in differentiation grade between biopsy and prostatectomy material found in the present study is in accordance with the data in the literature[10] and is probably due to intra-tumor variation. The recently proposed prognostic factor, based, among other clinical features, on the variation of nuclear shape, confirms the importance of intra-tumor heterogeneity.[30] Blom et al. (1990)[14] found variation in nuclear size of predictive value for survival. Both Partin[30] and Blom,[14] however, did not discriminate the locations of the different tumor areas in the prostate. This becomes particularly important regarding the anatomy of this organ.[34] Since capsular penetration occurs along nerve branches innervating the prostate postero-laterally, transition zone tumors, predominantly found in tissue removed for transurethral resection, are less frequently accompanied with growth outside the capsula and in general have a more favorable prognosis.[6,35,36] Moreover, there is reason to belief that besides anatomical differences, biological differences exist between peripheral and transition zone cancer. In 30 patients with stage-A or B disease, non-diploid tumor areas were found at lower tumor volume in nontransition compared to transition zone located tumors.[6] Likewise Greene et al. (1991)[6] we found aneuploid tumors located to the transition zone to be larger compared to peripheral zone cancers. However, the group of transition zone tumors was small and was overall of larger volume. This suggests that in our population the transition zone cancers represented a more progressed type of cancer compared to those transition zone tumors studied by Greene et al. (1991).[6]

Differences in innervation pattern, possibly accounting for variation in capsular penetration patterns between peripheral and transition zone tumors also apply for basal and apical parts of the prostate.[7] The short distance of the apical innervation plexus through the capsula causes earlier capsular tumor outgrowth in this part of the prostate compared to more basally located tumors.[7] Moreover, cancer found in prostates removed with radical cystectomy were predominantly located to the apex of the organ. The karyometric analysis of apical and basal tumor locations in the present study, revealed more irregular-shaped and larger nuclei in the locations in the apex. Tissue processing is probably not the reason for this finding, because normal prostate gland, adjacent to cancer, did not exhibit the karyometric differences. Since several studies found correlation of nuclear size[14] and shape[13,31] with prognosis, it is tempting to speculate that tumors located towards the apex may comprise a more aggressive

type. Although, this was not significant in our analysis the volume of tumors with positive margins at the apical resection area tended to be smaller than tumors invading the resection site at the bladder. It is of interest, however, that tumors invading the apical resection margin were significantly less heterogeneous in nuclear phenotype and had less irregular-shaped nuclei compared to cancers present in the bladder resection margin.

Besides differences in the basal-apical location of prostate cancer, nuclear shape values also varied in different locations in the equatorial plane of the prostate. Hence, prognostic value of nuclear shape, as indicated in some studies[13,31,32] may represent differences in tumor location within the prostate. Follow up data, in fact, are required to study this hypothesis. Nevertheless, these findings illustrate the need for the analysis of multiple tumor areas.

When the pathology prognostic markers were related to the karyometric findings, it became evident that several karyometric features correlated with more progressive tumor growth, independent of tumor location. Seminal vesical invasion and extracapsular tumor growth were both more common in heterogeneous tumors as described by variation in chromatin pattern between different tumor locations within the prostate. Karyometric heterogeneity was positively correlated with tumor volume, an important prognostic feature. In the logistic regression analysis, however, the karyometric analysis of nuclear heterogeneity was the most predictive characteristic for seminal vesical invasion and extracapsular tumor growth. In cancer not confined to the surgical specimen, the maximal value for nuclear shape irregularities (BEN) present in the different tumor locations was significantly higher than in tumors confined to the prostate. Heterogeneity of nuclear shape, however, within the tumor is considerable. Remarkably, variation in nuclear shape was the most powerful predictor of the presence of tumor in the surgical margins. In a logistic regression analysis, 79% of all cases was correctly classified in organ-confined or extra-prostatic disease based on the heterogeneity in chromatin pattern (Markovian feature L2) and the tumor volume.

When studying combinations of prognostic features it became apparent that capsular penetration was a relatively early event in cancer progression since most cases were found without one of the other markers present. With an increase in the number of positive markers, tumor heterogeneity in nuclear phenotype increased.

The findings of the present study confirm that nuclear shape irregularities are correlated with more progressed localized disease in prostate cancer. Analysis of

different tumor locations, however, revealed considerable intra-tumor variation. In particular tumors located to the apex showed increased nuclear shape irregularities. Whether this has prognostic implications remains unclear. Heterogeneity in karyometric features was overall the best predictor of local progression, independent of tumor volume.

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SUMMARY

The biology of tumors can be considered as a continuum ranging from low to high malignant potential. Although the extremes in this spectrum can be defined, for the vast majority of tumors no objective prognostic markers exist. Tumor stage and grade are now generally applied to predict the course of disease and select the most appropriate treatment in patients with urological cancer. In the molecular biology field, several genes have been liked to tumor behavior. The phenotypical tumor characteristics assessed in tumor grading, still provide important information on tumor malignancy. Unfortunately, low reproducibility and the lack of quantitative tumor markers render visual tumor grading less consistent. Hence, the cellular and nuclear features, used in the tumor grading criteria, have been quantified by image analysis techniques. In this thesis an outline is given of the available quantitative light microscopic analyses for the grading of urological cancers. Besides, our studies on the quantitative grading of bladder, renal cell, and prostate cancer are presented.

The **Introduction** (pp 9 - 56) gives an overview of the grading systems applied for prostate adenocarcinoma (prostate cancer), urothelial cell carcinoma (urinary bladder cancer), and renal cell carcinoma (kidney cancer). Furthermore, the available quantitative light microscopic techniques are described and the advantages and disadvantage are discussed. For each urological tumor the useful quantitative features are summed for tumor grading. Several conclusion are drawn after reviewing the literature:

1. Most studies comprise an analysis in research settings, where conditions are often standardized. Conclusions derived can not always directly be applied on routine practice.
2. Consequently, we consider standardization of preparation and image analysis routines of utmost importance.
3. Overviewing the literature several important features should be tested on their clinical applicability considering the results from research studies. For prostate cancer nuclear DNA content and possibly nuclear shape could be of additional value to routinely used tumor markers. Bladder cancer grading and especially follow up could be supported using DNA analysis of histological biopsies and quantitative analysis of (bladder washing) cytology. For renal cancer the yet routinely applicable methods are considered to be rare and more research oriented study is needed.

4. Further study on the use of quantitative light microscopy should include the following elements

- Clinical integration should be pursued for several features (see under 3).
- The logistic elements of such applications need further investigation.
- New approaches to quantitative grading should include tumor heterogeneity.

In the **first chapter** (pp 57 - 88) we describe the work on the quantitative grading of bladder cancer in both cytological and histological material. The optimal cytological preparation method is determined for nuclear size analysis and it is concluded that nuclear profile area in cytology can be useful for the grading of bladder cancer. In the second study we applied multivariate analysis for the grading of bladder cancer in histological plastic-embedded material. Its was concluded that multiple features could be more useful for tumor grading than nuclear profile area alone.

The **second chapter** (pp 89 - 105) describes the factors influencing reproducibility of quantitative analyses. In case of analysis of only small parts of the material, e.g. 50 nuclei, random selection of nuclei resulted in both low reproducibility and low correlation with tumor grade, whereas the application of selection criteria gave more consistent results. It should be beared in mind, however, that these criteria again introduce a subjective element in an initially objective intended analysis. Hence, in the second part of this chapter we report on the reproducibility of quantitative analysis of bladder washing material. Since more advanced image analysis techniques were available at this time we choose to trade the selection criteria for an increase in the number of analyzed nuclei. In the latter setup both reproducibility and correlation with clinical data was acceptable.

In the **third chapter** (pp 107 - 120) we applied a quantitative grading method in recurrent bladder cancer. Histological material from both the primary and first superficial tumor recurrence were karyometrically analyzed. The visual grading of neither the primary tumors nor the recurrences was useful for prediction of tumor progression during follow up. The karyometric features of the recurrent tumors, however, were useful for prediction of tumor progression, whereas the analysis of the primary tumor did not result in adequate prediction. It was concluded that karyometric features offer information beyond tumor grade. Moreover, karyometric analysis provided information on progressive changes in superficial cancer over time,

not detected by visual tumor grading but already indicative of later tumor progression.

A routine application of karyometric analysis is discussed in the **fourth chapter** (pp 121 - 138). In a pilot study two karyometric features were found useful for the prediction of presence of tumor in the bladder based on bladder washing material. The features also proved to correlate with tumor grade. To evaluate the routine applicability of a grading based on these features 1336 samples in five urological institutes were obtained and send to our laboratory. Karyometric analysis resulted in 90% sensitivity and 70% specificity for the detection of tumor. All carcinoma in situ cases could be detected. Moreover, the karyometric analysis correlated with clinical follow up in 787 patients indicating that the analysis provides early recognition of patients with potentially progressive cancer phenotypes using bladder washing.

The value of quantitative light microscopy in renal cancer is discussed in the **fifth chapter** (pp 139 - 154). Histological material of 121 patients is quantitatively analyzed with special reference to tumor heterogeneity. In a Cox multivariate analysis including both clinical, pathological, and karyometric features tumor stage appeared to be the most important prognostic factor. Karyometric features showed to have additional prognostic information. In particular, increased heterogeneity in nuclear size and chromatin patterns in different areas of the tumor were indicative of more malignant potential of the tumor.

In the **sixth chapter** (pp 155 - 167) we applied the karyometric approach described in chapter five for the prediction of tumor progression in patients with an initially low-stage renal tumor. Since no detectable metastases are present in these patients at time of diagnosis radical tumor nephrectomy would principally eradicate the disease. Heterogeneity in chromatin texture as assessed by analysis of several tumor areas was the best predictor of later tumor progression in a multivariate Cox analysis.

The **seventh chapter** (pp 169 - 187) describes the use of karyometric analysis in prostate cancer. Karyometric analysis was correlated to several clinical features and so-called pathological progression markers (seminal vesical invasion, extracapsular tumor growth, positive resection margins, and lymph node metastases). Nuclear shape irregularities, earlier found to be a bad prognostic indicator, were more pronounced in tumors located to the apex of the organ. Heterogeneity in karyometric features

(chromatin pattern, DNA content, and nuclear shape) was significantly more abundant in cancers not confined to the prostate compared to organ-confined disease. This was independent of tumor volume, an important prognostic marker.

The studies in this thesis comprise the application of quantitative light microscopy in research as well as clinical settings in urological oncology. As such, the technique can be supportive in the routine clinical diagnosis of bladder wash cytology and renal cell cancer. For prostate adenocarcinoma the data are merely of scientific relevance. Future analysis of prostate biopsy material may be a field of application.

SAMENVATTING

De maligniteit van tumoren kan beschouwd worden als een continuum, varierend van laag naar hooggradig kwaadaardig. Alhoewel de uitersten in dit spectrum vaak te definieren zijn, bestaan er geen duidelijke objectieve prognostische factoren voor het meerendeel van de tumoren. Tumor graad en stadiering zijn in de klinische praktijk de meest belangrijke factoren en bepalend voor de therapie keuze. Recent moleculair biologisch onderzoek toonde een verband tussen bepaalde genen en tumorgedrag. Phenotypische tumor karakteristieken worden echter alom gehanteerd in tumor gradering. De lage reproduceerbaarheid en de onmogelijkheid van quantificering maken tumor gradering minder aantrekkelijk. Beeldanalyse van cel en celkern kenmerken in licht-microscopische beelden maakt objectivering van tumorgradering mogelijk. In dit proefschrift wordt een review gegeven van de literatuur op het gebied van de quantitatieve gradering van urologische tumoren middels karyometrie (de quantificering van celkern kenmerken). Tevens worden enkele studies van de quantitatieve gradering van blaas, nier, en prostaat kanker beschreven.

De **introductie** (pp 9 - 56) geeft een overzicht van de visuele graderings criteria voor de gradering van prostaat, blaas en nier tumoren. Bovendien worden de beschikbare parameters en de voor- en nadelen van quantitatieve licht-microscopie beschreven. Na review van de tot nu toe gepubliceerde studies leidde tot enkele conclusies:

1. De meeste studies omvatten patienten populaties en technieken in onderzoeks opstellingen. De gebruikte technieken waren echter niet gelijk in iedere studie waardoor vergelijking van resultaten niet altijd mogelijk is.
2. Standardisatie van materiaal bewerkingen en metingen is een eerste vereiste voor een implementatie van beeldanalyse in de klinische praktijk.
3. Uit de studies bleken enkele parameters van nut en dienen deze te worden geintegreerd in verdere studies. Bij prostaat tumoren waren DNA-inhoud en mogelijk celkern vorm parameters met prognostische waarde. Blaas tumor gradering en met name de follow-up van patienten met blaas kanker kan worden ondersteunt met DNA-histogram analyse in bijvoorbeeld (blaasspoeling) cytologie materiaal. Onderzoek naar quantitatieve licht-microscopie van niercel tumoren heeft nog niet geleid tot een evident klinisch voordeel van deze techniek. Wel bestaat er correlatie tussen enkele parameters en tumor gradering.

4. Verder study naar quantitatieve licht-microscopische parameters in urologische oncologie dient de volgende elementen te bevatten:
   - Klinische integratie van de gevonden parameters.
   - De logistieke elementen behoeven nadere analyse.
   - Tumor heterogeniteit dient geimplementeerd in de analyse.

In het **eerste hoofdstuk** (pp 57 - 88) worden de studies beschreven naar de quantitatieve gradering van cytologisch en histologisch materiaal van urotheelcel tumoren van de blaas. De optimale cytologische preparatie techniek werd bestudeerd en celkern oppervlakte in cytologisch materiaal was een goede maat voor visuele tumor graad. In een tweede studie in dit hoofdstuk werd plastic ingebed weefsel gebruikt voor de quantitatieve gradering van blaastumoren. Combinatie van meerdere parameters resulteerde in een betere correlatie gradering met visuele tumor graad.

Het **tweede hoofdstuk** (pp 89 - 105) beschrijft de factoren van invloed op de reproduceerbaarheid van quantitatieve analyses. Analyse van slechts een klein aantal willekeurig geanalyseerde celkernen (50) per sample resulteerde in een lage reproduceerbaarheid en lage correlatie waarden tussen kerngrootte en visuele tumor graad. Na gebruik van selectie criteria verbeterde zowel de reproduceerbaarheid als de voorspelling van tumor graad. Wel dient hier vermeld dat het hanteren van selectie criteria een subjectief element in de analyse betekend in een in opzet objectieve gradering. Vandaar dat in de tweede studie in dit hoofdstuk een groter aantal celkernen werd geanalyseerd per sample. De analyse van 100 of meer celkernen in blaasspoelingen resulteerde in acceptabele reproduceerbaarheid en goede correlatie met histologische tumor graad.

In het **derde hoofdstuk** (pp 107 - 120) wordt de toepassing van quantitatieve licht-microscopie in recidiverend oppervlakkig urotheelcel carcinoom beschreven. Histologisch materiaal van zowel de primaire als de eerste recidiverende tumor werden karyometrisch onderzocht. Visuele gradering, noch van de primaire tumor, noch van de recidief tumor verspelden het optreden van latere tumor progressie. The karyometrische parameters van de recidiverende tumor waren echter voorspellend voor het optreden van tumor progressie. Wij concludeerden dat karyometrische analyse een betere prognostische factor is dan tumor graad. Voor de voorspelling van tumor progressie is nauwkeurige follow-up van recidief tumor echter noodzakelijk.

Een routinematige toepassing van karyometrische analyse wordt beschreven in het **vierde hoofdstuk** (pp 121 - 138). In een kleine pilot studie worden twee karyometrische parameters gevonden voor voorspelling van tumor in de blaas aan de hand van analyse van blaasspoelings materiaal. Tevens zijn deze parameters bruikbaar voor de gradering van de tumor. In een grote populatie van 1336 blaasspoeling samples, verzameld in vijf urologische klinieken worden de gevonden parameters getest. De sensitiviteit van de karyometrische test was 90% terwijl een specificiteit van 70% gevonden wordt. De lage specificiteit wordt mede verklaard door de hoge predictieve waarde voor tumor recidieven. Van alle tumor recidieven had 86% een afwijkende karyometrische score in het sample voor het tumor recidief. Tevens werden in deze studie de logistieke aspecten van routinematige karyometrische analyse bestudeerd.
De resultaten van deze studie impliceren dat de voorgestelde sample versturing en karyometrische analyse een routinematig toepasbare, methode van follow-up van patienten met een oppervlakkig blaas carcinoom.

Karyometrische analyse van niercel tumoren is beschreven in het **vijfde hoofdstuk** (pp 139 - 154). Histologisch materiaal van 121 patienten werd geanalyseerd met speciale aandacht voor tumor heterogeniteit. In een Cox multivariate analyse, waarin klinische, pathologische en karyometrische parameters gezamenlijk werden geanalyseerd bleek de karyometrische analyse additionele waarde te hebben voor de voorspelling van overleving. Met name karyometrische parameters die heterogeniteit in celkern grootte en chromatine patroon beschreven waren van prognostische waarde.

In het **zesde hoofdstuk** (pp 155 - 167) werd karyometrische analyse gebruikt voor de voorspelling van tumor progressie in patienten met gelocaliseerd niercel carcinoom die middels tumor-nefrectomie in opzet curatief werden behandeld. Veertig procent van de patienten ontwikkelde tumor progressie tijdens follow-up. Tumor heterogeniteit zoals geobjectiveerd middels karyometrie was de meest belangrijke prognostische factor in deze populatie.

Het **zevende hoofdstuk** (pp 169 - 187) beschijft het gebruik van karyometrie in de analyse van verschillende tumor locaties in prostaat adenocarcinoom. Karyometrische parameters bleken gecorreleerd te zijn met verschillende pathologische kenmerken

van tumor progressie (vesicula seminalis ingroei, extracapsulaire tumor groei, tumor in het resectievlak en lymfklier metastasen). Celkern vorm onregelmatigheden waren meer frequent aanwezig in tumors gelocaliseerd in de apex van de prostaat. Wederom bleek heterogeniteit in karyometrisch analyse een belangrijke factor samenhangend met extracapsulaire tumor groei. Dit bleek onafhankelijk van de tumor gradering en tumor grootte.

De studies in dit proefschrift toonden aan dat karyometrische analyse informatie verschaft over urologische tumoren die additioneel is aan de visuele tumor graderings systemen. Bovendien kan de techniek inzicht geven in het groei verloop van tumoren. Gezien de toegenomen ontwikkelingen op het gebied van de beeldanalyse apparatuur vormt de methode een routinematig bruikbare ondersteuning van de klinische en patholoog-anatomische beslisneming in de uro-oncologie.

Curriculum Vitae

De auteur van dit proefschrift werd op 9 maart 1965 geboren te Rijswijk (ZH). De middelbare school werd gevolgd in Leiden (Visser 't Hooft Lyceum) en afgesloten met het eindexamen VWO in 1982. De studie geneeskunde werd gestart in 1983 aan de Rijksuniversiteit te Leiden. Tijdens de studietijd werd een student assistentschappen gelopen in de uropathologie, o.l.v. dr. Ooms en dr. Boon in het West Einde Ziekenhuis Den Haag en het Leids Cytologisch en Pathologisch Laboratorium. Tevens deed hij wetenschappelijke onderzoek op het gebied van de neurochirurgie in een stage van een half jaar aan de dept. of Neurosurgery, Medical College of Virginia, Richmond, Virginia o.l.v. dr. Muizelaar. Het artsexamen werd behaald in 1990. Het onderzoek op het gebied van de uropathologie werd voortgezet in het Urologisch Research Laboratorium van de afdeling Urologie, St. Radboud Ziekenhuis, Nijmegen. Sinds april 1993 is hij in de chirurgische vooropleiding tot uroloog in het St Elisabeth Ziekenhuis, Tilburg.

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Stellingen behorende bij het Proefschrift

QUANTITATIVE LIGHT MICROSCOPY IN UROLOGIC ONCOLOGY

Quantitatieve interpretatie van objecten in licht-microscopische beelden ondersteunt, meer dan vervangt, de opinie van de patholoog-anatoom.

Het groot aantal criteria in de visuele licht-microscopische gradering van tumoren verminderd de reproduceerbaarheid van deze methode.

Zolang evidente basale oorzakelijke factoren van kanker onbekend zijn is iedere gradering slechts te baseren op empirisch gevonden 'tekenen' van maligniteit.

Quantitatieve licht-microscopie vormt een bruikbare methodiek voor de objectivering van phenotypische heterogeniteit van tumoren.

Standardisering van materiaal verwerkingsmethoden en beeld analyse technieken is een eerste vereiste voor de routinematige toepassing van karyometrische analyse.

Prognose voor kanker patienten is afhankelijk van drie factoren: 'the response of the patient, the commissions and omissions of the physician, and the potentialities of the neoplasm'.
(Mostofi, Cancer Chemother. Rep., 59: 111, 1975)

Tumor heterogeniteit is een belangrijke prognostische factor bij het niercelcarcinoom.

De anatomische locatie van maligne ontaarde cellen binnen de prostaat is van prognostische betekenis.

De vaak iets geringschattende houding ten aanzien van vroeger wetenschappelijk onderzoek zou een positieve bijdrage aan het relativerend wetenschappelijk bewustzijn kunnen vormen.

De steeds verdere etherdilutie in de vorm van een toenemend getal in televisie zendkanalen doet een homeopathische grondgedachte vermoeden.

Zwaartekracht kan worden opgevat als de oppervlakte spanning van de aarde: het verlaagd bergen en doet diepten dichtslippen.

Een basisinkomen als maatschappelijke vluchtstrook vergemakkelijkt het invoegen maar verminderd niet de kans op panne.

Het houden van vee en kweken van gewassen zijn sinds mensenheugenis geaccepteerde vormen van indirecte genetische manipulatie.

H.G. van der Poel
januari 1994.